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In order to understand how the tumor suppressor p53 blocks the cell-cycle at the G1-S transition we studied how p21, a transcription target of p53, interacts with and inhibits cyclin-dependent kinases (cdk). We identified specific cyclin-binding Cy motifs on the p21 molecule that were essential for docking p21 on cyclin-cdks and discovered that such Cy motifs were present and functional on substrates and activators of cdks. This year we discovered the presence of a Cy motif on a newly identified human DNA replication initiation factor, Cdc18. This Cy motif is required for the stable association of Cdc18 with cyclin A-cdk2 in vivo. Wild type Cdc18 is in the nucleus at G1 and removed from the nucleus at S phase once cyclin-cdks are activated. The Cy motif of Cdc18 is necessary for this S phase specific removal of nuclear Cdc18 implying that Cdc18 is a physiological substrate of cyclin-cdks at the G1-S transition.

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This contract supports the salary of the principal investigator.

INTRODUCTION

The original aims of this contract were met in 1996, when our results strongly suggested that the main growth suppressive action of p53 derives from its ability to activate transcription rather than binding to and inhibiting RPA (1). We turned our attention to studying one of the major effectors of p53, a protein variously called p21/WAF1/Cip1. p53 induces the mRNA for this gene, p21 protein product increases and it suppresses cell growth (2, 3). Failure to induce p21 (due to mutations in p53) abrogated the G1-S block following DNA damage that is necessary to repair damaged DNA before the mutations are fixed by replication. Acquisition of new mutations is now recognized as a hallmark of cancer development and progression.

We therefore spent time understanding how p21 stops cell growth at the G1-S transition. In the 1996 report I introduced the cell-cycle and how it is regulated by the periodic appearance of cyclin-cdk activity. The crucial activity at the G1-S transition is the activation of cyclin E-cdk2 and cyclin D-cdk4. During S phase, cyclin A-cdk2 begins appearing. All three activities are essential for the progression of the cell-cycle.

We showed that the regions of p21 involved in interacting with and inhibiting (i) the cyclin -cdk kinases and (ii) PCNA are separable from each other (4). The N terminal domain of p21 (p21N) interacts with the cdk2 protein and inhibits cyclin-cdk kinase activity, while the C terminal domain (p21C) interacts with and inhibits PCNA. Using these separated domains we showed that p21N inhibits growth of transformed human osteosarcoma cells, SaOs2 suggesting that the minimal requirement for growth suppression by p21 is its ability to inhibit the cyclin-cdk kinases. We also discovered that p21 uses two separate motifs, a cyclin binding Cy motif and a cdk binding K motif which independently bind to the regulatory cyclin subunit and the catalytic cdk subunit (5). Both these motifs and interactions are essential for optimal kinase inhibition and cell growth suppression.

The importance of the Cy motif of p21 in interacting with the cyclin -cdk complex was highlighted by our discovery that a 12 amino acid peptide containing this Cy motif could competitively block the association of cyclin-cdk with p21 (5). The X-ray crystal structure of p27 (a member of the p21 family of cdk inhibitors) suggests that the Cy motif of p21 interacts with the MRAIL motif of cyclins and the interaction is independent of a more complicated interaction between the K motif and the catalytic cdk (6). Yet, although both the Cy-cyclin and K-cdk2 interactions are essential for optimal kinase inhibition, the Cy-cyclin interaction (interrupted by the Cy motif containing peptide) appears to be the primary docking interaction which then allows the K-cdk2 interaction to take place. If this docking interaction is blocked then the cyclin-cdk could be partially immunized to the inhibitory action of p21.

We also have evidence from immunocytochemical studies of breast cancers (another project, not supported by the USAMRDC) that p21 is up-regulated in 33% of breast cancers (relative to breast epithelium). In 10% of the cancers nuclear p21 levels are maximal (among all breast cancers studied) but proliferate as though they are resistant to the molecule. No mutations were found in the p21 gene of several hundred tumors (including breast cancers) analyzed (7). Therefore the high frequency of apparent resistance to p21 (10%) cannot be explained by genetic mutations inactivating p21. Based on our observation of the critical role of the Cy motif-Cyclin interaction for kinase inhibition, we hypothesized that a cdk activator (cdk activating kinase CAK or Cdc25) may occupy the MRAIL site on the cyclin and prevent p21 from binding to cyclins and inhibiting the cdk kinase. Cellular molecules such as these activators could be up-regulated in the breast cancers and impair the action of p21. This would be a novel mechanism by which the cdk inhibitors are bypassed. In addition, a p21-mimetic chemical may be even more active in this sub-set of breast cancers because it prevents the activation of cyclin-cdks by such cdk activators.

Last year we identified a cyclin binding motif near the N terminus of Cdc25A, an activator of cdks, that is similar to the cyclin binding Cy (or RXL) motif of p21/CIP1 family of cdk inhibitors and separate from the catalytic domain (8). Mutations in this motif disrupted the

association of Cdc25A with cyclin E- or cyclin A-cdk2 *in vitro* and in *vivo* and selectively interfered with the de-phosphorylation of cyclin E-cdk2. A peptide based on the Cy motif of p21 competitively disrupted the association of Cdc25A with cyclin-cdks and inhibited the de-phosphorylation of the kinase. p21 inhibited Cdc25A/cyclin-cdk2 association and the dephosphorylation of cdk2. Conversely, Cdc25A, which is itself an oncogene up-regulated by the Myc oncogene, associated with cyclin-cdk and protected it from inhibition by p21. Cdc25A also protected DNA replication in Xenopus egg extracts from inhibition by p21. These results described a mechanism by which the Myc- or Cdc25A-induced oncogenic and p53- or TGFβ-induced growth-suppressive pathways counterbalance each other by competing for cyclin-cdks.

Cdc18/CDC6: Several proteins essential for the initiation of DNA replication have been identified initially in *S. cerevisiae.* Homologs of these proteins have been identified in humans and in other eukaryotes , where they are expected to play an equally important role in DNA replication. Some of these proteins will have to be activated at the G1-S transition by cyclin-cdk phosphorylation , and several of them promise to be good target for development of inhibitory molecules that will inhibit cancer cell proliferation.. CDC6 in *S. cerevisiae* and Cdc18 in *S. pombe*, is one such molecule that is essential for the onset of DNA replication. Xenopus and yeast CDC6/Cdc18 and ORC are required for DNA replication and for the loading of the MCM proteins onto the chromatin. After mitosis, synthesis of new CDC6/Cdc18 protein allows the loading of MCMs on the chromatin (9). The pre-replication initiation complex thus formed in G1 is ready to initiate DNA replication upon the activation of S phase promoting factors like cyclin-cdks and CDC7 kinase. Once replication has initiated the removal of CDC6/Cdc18 during S phase (in yeast) prevents the re-loading of MCM proteins on the chromatin, thereby preventing re-replication of DNA.

We recently identified and cloned a human protein homologous to yeast CDC6/Cdc18 (hCdc18) (10). Although the rapid and total destruction of yeast CDC6/Cdc18 protein in S phase is a central feature of yeast DNA replication, the total level of hCdc18 protein is unchanged throughout the cell-cycle. Epitope tagged hCdc18p is nuclear in G1 and cytoplasmic in S phase cells, suggesting that DNA replication may be regulated either by the translocation of hCdc18 protein between nucleus and cytoplasm or the selective degradation of the protein in the nucleus. hCdc18p also associates directly with cyclin-cdks through the cyclin subunit an interaction that is seen in vivo and in vitro. Human Orc1 protein associates with hCdc18p in vitro and in vivo , consistent with the role of the latter in the control of human DNA replication.

The interaction of hCdc18 with cyclin-cdks and the displacement of hCdc18 from the nucleus to the cytoplasm at the G1-S transition led us to speculate whether hCdc18 was a substrate of cyclin-cdks at the G1-S transition. Based on the hypothesis that several substrates and regulators of cyclin-cdks that stably associate with the kinase have Cy motifs, we extended our studies to address whether hCdc18 had functionally important Cy motifs.

BODY

SPECIFIC AIM FOR YEAR 4

Year 4 (1997-'98)

Mutagenize the Cy motif of p21 to replace all the amino acids. Synthesize the mutant p21 proteins to determine if they bind to cyclins and inhibit cdk. These experiments address the physical constraints for amino acid side chains of a functional Cy motif (Task 5).

In the course of the year we added a new aim: is the Cy motif a generalizable motif that can be used to recognize physiological substrates of cyclin-cdks? Specifically, hCdc18, a human DNA replication factor associates with cyclin-cdks in vivo. Does it do so through Cy motifs, and is that interaction important for the physiology of the protein.?

METHODS

Expression of bacterial proteins, baculovirus expressed cyclin-cdks and in vitro kinase assays: Described in earlier reports.

Mutagenesis: Restriction enzyme sites AgeI and SpeI were introduced on either side of the ACRRLFGPVD motif of p21 by PCR directed mutagenesis. A 70 mer that encoded these amino acids and flanking sequences and that was compatible to the unique enzyme sites on either side was made with degenerate nucleotides at sites corresponding to RRLF so that any of three amino acids (including wild type) could be encoded at these positions. 18 nucleotide long oligomers that perfectly match the degenerate 70 mer at either end, were used in a PCR reaction (with the degenerate oligonucleotide as template) to produce a library of duplex oligonucleotides that can be cut with AgeI and Spe I and inserted into the p21 containing plasmid.

Two-hybrid assay for the interaction of the Cy1 motif of p21 with cyclin E: The Open reading Frame of the cyclin E cDNA was cloned into pAS2 to produce pAS2-Cyclin E , a plasmid that expressed in yeast Y190 the GAL4 DNA binding domain fused to cyclin E (11). The N terminal 90 amino acids of p21 (containing only the Cy1 and K motifs, and eliminating the redundant Cy2 motif at the C terminus of p21) was expressed in Y190 fused to the GAL4 activation domain from a plasmid pACT-p21N. Y190 transformed with pAS2-Cyclin E (TRP1+) and pACT-p21N (LEU2+) were assayed for interaction between cyclin E and p21N in the two-hybrid assay. If they interacted, the yeast would be lacZ+ (blue on X-gal plates) and HIS+ (grows on plates lacking histidine).

Immunofluorescence. U2OS cells were transfected with pAHP-Cdc18 or with pA3M-Cdc18 plasmids that express CDC18 in mammalian cells tagged with an hemagglutinin epitope or a myc epitope respectively (10). Cells were fixed in 3% formaldehyde containing 2% sucrose at room temperature for 10 minutes, washed twice with PBS and permeabilized in Triton solution (3% bovine serum albumin, 0.5% Triton X-100 in PBS) for 5 minutes. After washing with PBS, cells were incubated with anti-HA antibody (12CA5) in hybridoma culture supernatant diluted 1:100, anti-Myc monoclonal in mouse ascites diluted 1:100 or anti-p21 rabbit polyclonal antibody (Santa Cruz) diluted 1:100 for 20 minutes at 37°C. Cells were then washed three times with PBS and incubated with the secondary antibody (FITC conjugated goat anti-mouse IgG or rhodamine conjugated goat anti-rabbit IgG, Jackson laboratory). After washing three times with PBS, cells were stained briefly with DAPI and photographed under a fluorescent microscope.

Pull-down assay: Affinity chromatography on glutathione beads coated with various GST-fusion proteins ("pull-down" assay) were done as described (5, 10).

RESULTS

Mutagenesis of Cy1 motif of p21

This novel strategy was adopted because we hoped to examine the effects of single and multiple amino acid substitutions in the core motif in an economical manner. Unfortunately, in practice, the strategy resulted only in frame-shift mutations. After trying different condition of PCR and sequence variations we abandoned this approach. In the coming year we will adopt the more expensive and labor intensive course of synthesizing different sets of oligonucleotides to

introduce the mutations we desire in GEXp21N. We hope to make about 40 different mutant Cy motifs and test their efficacy biochemically.

Two-hybrid assay of Cy1-cyclin E interaction

Having failed at economically making a library of Cy motif variants, we attempted another strategy where we could make a library of mutant p21 molecules by error-prone PCR and then use a selection and a screen for the mutants we desired. If the interaction of Cy1 and cyclin E could be recapitulated in a yeast two-hybrid assay, we planned to employ a negative selection strategy to screen for mutants in the Cy motif that decreased its effectiveness at interacting with the Cyclin (12). Briefly, the two-hybrid interaction would have been reproduced in a yeast strain where transcription activation of an URA3 gene results in toxicity in 5-FOA (Fluoro-orotic acid). One could then mutagenize the Cy motif, identify colonies that grow up on 5-FOA plates and sequence the mutant Cy motifs for mis-sense mutations. Simultaneously using the original Y190 transformed with pAS2-Cyclin E and pACT-p21N (with mutations) we planned to employ a screening strategy to look for mutants in the Cy motif that still allow the two-hybrid interaction (produce blue colonies on X-gal). Sequencing these mutants would identify amino acids that are tolerated at various positions in the Cy motif.

The two-hybrid interaction between p21N and cyclin E did not produce a positive result (LacZ was not activated) forcing us to abandon this approach. One possibility for the failure could be that the GAL4 activation domain fusion at the N terminus of p21N makes the Cy1 motif of p21N non-functional for interacting with cyclin E. To address this possibility we plan a two-hybrid interaction once again with full length p21 and cyclin E. As published, full length p21 has a second Cy motif, Cy2, near the C terminus which could be functional in interacting with cyclin E in a two-hybrid assay. If this strategy works, then we will mutate the Cy2 motif of p21 and perform the selection and screening strategies described above to determine what constitutes a Cy motif.

Stable complex formation between cyclin-cdks and Cdc18

Since Cdc18p and cdc2 associate with ORC in *S. pombe* cells, and a direct interaction has also been reported between *S. cerevisiae* CDC6 and CDC28, we tested whether human Cdc18p associated with any of the cyclin-cdk kinases. Cyclins D1, D2, D3, E, A, or B produced by in vitro transcription-translation in a rabbit reticulocyte lysate associated well with GST-Cdc18p produced in bacteria (Fig. 1; compare with interaction with the positive control, p21). Cdk2 produced by in vitro transcription-translation did not associate with bacterially produced GST-Cdc18p (Fig. 1). Therefore, Cdc18 associates with the cyclin subunit of cyclin-cdk in vitro.

In vivo association of hCdc18 with Orc1 and Cyclin/Cdk2.

To demonstrate the association of Cdc18p with cdk2 and with Orc1p in vivo, a plasmid expressing GST-Cdc18p was transiently transfected into human kidney 293T cells (Fig. 2). Negative controls included cells transfected with plasmids expressing GST and GST fused to an irrelevant protein. Selective precipitation of GST-Cdc18p from the cell extracts showed co-precipitation of cyclin A, cdk2 and Orc1p, confirming that the cyclin-cdk2/Cdc18p and Orc1p/Cdc18p associations are observed in cells. The addition of ethidium bromide to the reactions did not affect the interactions (Fig. 2, lane 3), implying that the interactions are not mediated through DNA. Cdc18 associates with cyclin A-cdk2 in vivo.

Subcellular localization of hCdc18 protein changes during cell cycle.

We expressed Cdc18 tagged with an N terminal HA-epitope by transient transfection of human U2OS osteosarcoma cells and immunostained with anti-HA antibody (12CA5). The results were confirmed with Cdc18 fused to a C terminal myc epitope. Of 100 cells expressing epitope tagged-Cdc18, 20-40% expressed the protein in the cytoplasm and 80-60 % in the nucleus in different experiments (Fig. 3a and b). 30% of the cells were in G1 as judged by positive staining for nuclear p21. In all these G1 cells HA-hCdc18p was detected in the nucleus (Fig. 3a). All cells where HA-hCdc18p was found in the cytoplasm were negative for p21 (Fig. 3a), implying that HA-hCdc18p was seen in the cytoplasm in some phase other than G1.

To further clarify when during the cell cycle hCdc18p appears in the cytoplasm, we expressed myc-epitope tagged hCdc18 protein in U2OS cells and stained the cells with anti-myc and anti PCNA antibody (Fig. 3b). Among the 120 cells expressing Myc-hCdc18, 30-40% were

in S phase as judged by nuclear staining for PCNA. Myc-hCdc18p was detected in the cytoplasm in all these cells (Fig. 3b). Whenever, myc-hCdc18p was detected in the nucleus, the cells were negative for PCNA and were therefore not in S phase. Therefore, hCdc18 protein is present in the nucleus during G1 phase (p21 positive) in time to form pre-replication initiation complexes. After cyclin/cdks become active in S phase (PCNA positive), hCdc18p is exported to the cytoplasm, to come back to the nucleus some time before the next G1. Thus, although the total hCdc18 protein amount is unchanged during the cell cycle (10), the activity is likely to be regulated by changes in its subcellular localization which could be related to the activation of cyclin-cdk at the G1-S transition.

Cdc18 contains a putative cyclin binding motif on the N-terminus similar to Cy1 motif of $p21^{CIP1}$.

p21 has two cyclin binding motifs, Cy1 (amino acid 17-24) and Cy2 (amino acid 152-158). p27, p57, p107, p130, E2F1 and Cdc25A use a similar motif to interact with a cyclin. The important amino acid residues in the motif are RRLFG. A 12 amino acid long peptide (PS100) derived from the Cy1 region of p21 interrupts the interaction between p21 and cyclin E or cyclin A (5).

Interestingly, human Cdc18 protein contains a similar amino acid sequence at residues 94-98 (RRLVF) and we hypothesized that this sequence is used for the interaction with cyclins. To test this we investigated the effect of PS100 on the association of Cdc18 with cyclins. Fig. 4 shows that when pull-down assays were carried out with GST-Cdc18 and ^{35}S-labeled cyclin A in the presence of PS100 peptide, the Cdc18-cyclin interaction was inhibited. A mutant peptide PS101, where LF residues are changed to KK, did not affect the interaction. Therefore, a Cy1 like motif was important for the interaction of Cdc18 with Cyclin A.

Mutation in the putative Cy motif of Cdc18 affects its cyclin binding activity.

To confirm whether the RRLVF sequence on Cdc18 is responsible for binding to cyclins, we changed the LV residues to KK by site directed mutagenesis (Cdc18ΔCy1). 293T cells were transfected with plasmids expressing Cdc18 or Cdc18ΔCy1 as GST fusion proteins (Fig. 5). The expressed GST-Cdc18 proteins were affinity purified from the cell lysate with glutathione agarose beads. The associated cyclin A and cdk2 were detected by Western blot analysis. Although equal quantities of GST (from the EBG transfection in lane 1; visible in the 30 kD area of the gel, not shown in the Figure), GST-Cdc18 (lane 2) and GST-Cdc18ΔCy1 (lane 3) were expressed and isolated on the glutathione agarose beads, cyclin A and cdk2 could only associate with GST-Cdc18. This observation confirms the importance of the RRLVF motif for association of Cdc18 with cyclin A-Cdk2 in cells.

Cy motif of Cdc18 required for cytoplasmic displacement of protein in S phase.

Cdc18ΔCy1 was studied to analyze the contribution of the Cy motif to the localization of epitope-tagged Cdc18 in asynchronous cultures (Table below). Mutation in the Cy motif resulted in quantitative retention of Cdc18 in the nucleus. A Cdc18 derivative with a mutation in the putative nucleotide-triphosphate (NTP) binding motif did not show the constitutive localization of the protein in the nucleus and serves as a negative control.

EFFECT OF POINT-MUTATIONS ON LOCATION OF Cdc18 IN ASYNCHRONOUS CELLS
(percentage of cells with indicated distribution of epitope tagged Cdc18p).

Location of Cdc18	Nuclear	Cytoplasmic	Nuclear+Cytoplasmic
wild-type	59%	36%	5%
ΔCy	91%	6%	3%
NTP binding site mutated (negative control)	25%	61%	15%

Flow cytometry of cells transfected with the mutant versions of Cdc18 and a plasmid expressing GFP (as a marker for transfected cells) showed that over-expressed mutant Cdc18 did not block the cells in G1 (data not shown). Therefore Cdc18ΔCy1 is constitutively nuclear even in cells that have activated cyclin-cdk and entered S phase.

We conclude that the Cy motif of Cdc18 is indeed functional and that the cyclin-cdk's interaction with the over-expressed CDC18 is essential for the protein to be displaced from the nucleus at the onset of S phase. We are currently testing whether the phosphorylation of Cdc18 by cyclin-cdks is responsible for this displacement.

FIGURE LEGENDS

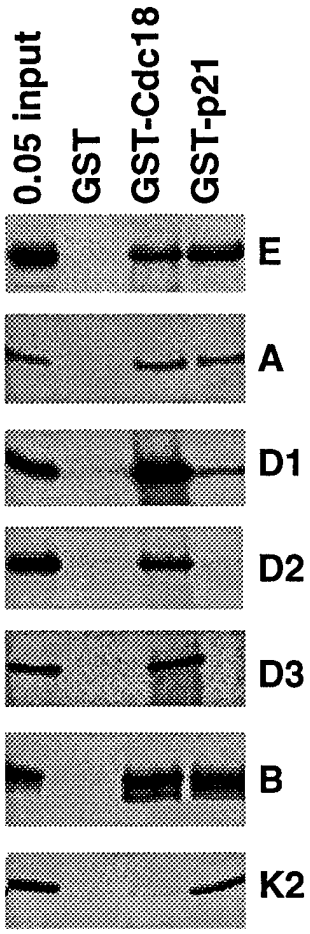
Fig. 1 Cdc18p associates with cyclin-cdks through the cyclin and the association is regulated by a mitotic inhibitor. Indicated S^{35} labeled proteins were produced by in vitro transcription-translation and tested for association with bacterially produced GST, GST-Cdc18 and GST-p21. 0.05 input: 5% of the input protein incubated with indicated glutathione agarose beads. Proteins tested were Cyclins E, A, D1, D2, D3 and B and cdk2 (K2).

Fig. 2. Association of Cdc18p with Orc1p, cyclin A and cdk2 in vivo. 293T cells were transfected with pEBG (lane 1), pEBG-Cdc18 (lanes 2 and 3) or pEBG-Cdc18rev (lane 4). Cellular proteins associated with glutathione-agarose beads were detected by immunoblot analyses. Top three panels: lysates input into the reactions were detected by anti-Orc1p (Orc1), anti-cyclin A (Cyc A) and anti-cdk2 (cdk2). Bottom four panels: proteins associated with glutathione agarose beads were detected immunoblot analyses. To see the effect of ethidium bromide on the interaction, 50 μg/ml of ethidium bromide was added to the extract, incubated for 30 minutes on ice, centrifuged to remove any particulate matter and the resulting extract incubated with glutathione agarose beads (lane 3).

Fig. 3 Subcellular localization of epitope tagged hCdc18p. **(A)** Nuclear hCdc18p is seen in G1 cells that are positive for nuclear p21. Asynchronously growing human osteosarcoma cells U2OS were transfected with plasmid expressing HA-Cdc18p and two days after transfection the cells were stained with DAPI (Top row); anti-HA monoclonal antibody (12CA5) and FITC-conjugated goat anti-mouse secondary antibody (middle row); and anti-p21(CIP1) rabbit polyclonal antibody and rhodamine-conjugated goat anti-rabbit secondary antibody (bottom row). First two columns on left, positive nuclear staining for p21 (G1 cells) ; last two columns on right, negative staining for p21 (non G1 cells). **(B)** S phase cells marked by nuclear PCNA have Cdc18p in the cytoplasm. Asychronously growing U2OS cells were transfected with plasmid expressing myc-Cdc18p and the cells stained with DAPI (top row), anti-myc monoclonal antibody and FITC conjugated secondary antibody (middle row) and auto-immune human serum containing anti PCNA antibody and rhodamine conjugated secondary antibody (bottom row).

Fig. 4 The association of cyclin A with Cdc18 is competitively blocked by a Cy motif containing peptide. ^{35}S methionine labeled cyclin A was produced by in vitro transcription-translation (lane 1, 1/20th of cyclin A input into the other reactions) and incubated with indicated GST-fusion proteins in the absence or presence of peptides containing the Cy1 motif of p21 (PS100, lane 4) or a mutant peptide that has two residues of the Cy motif changed (PS101, lane 5). Cyclin A bound to the GST-fusion proteins are visualized in lanes 2 to 5.

Fig. 5 The association of Cdc18 with cyclin-cdks in vivo is disrupted upon mutation of the Cy motif of Cdc18. Plasmids EBG (lane 1) , EBG-Cdc18 (lane 2) and EBG-Cdc18ΔCy1 (lane 3) were transfected into 293T cells and the expressed GST-fusion proteins isolated (along with associated cellular proteins) on glutathione agarose beads. One-tenth of input proteins (top two panels) and proteins associated with glutathione agarose beads (bottom three panels) were visualized by immunoblotting with appropriate antibodies. In the third panel Lane 1 contains an intense 30 kD GST protein that is not visible in the figure (which only shows the 90 kD region containing GST-Cdc18).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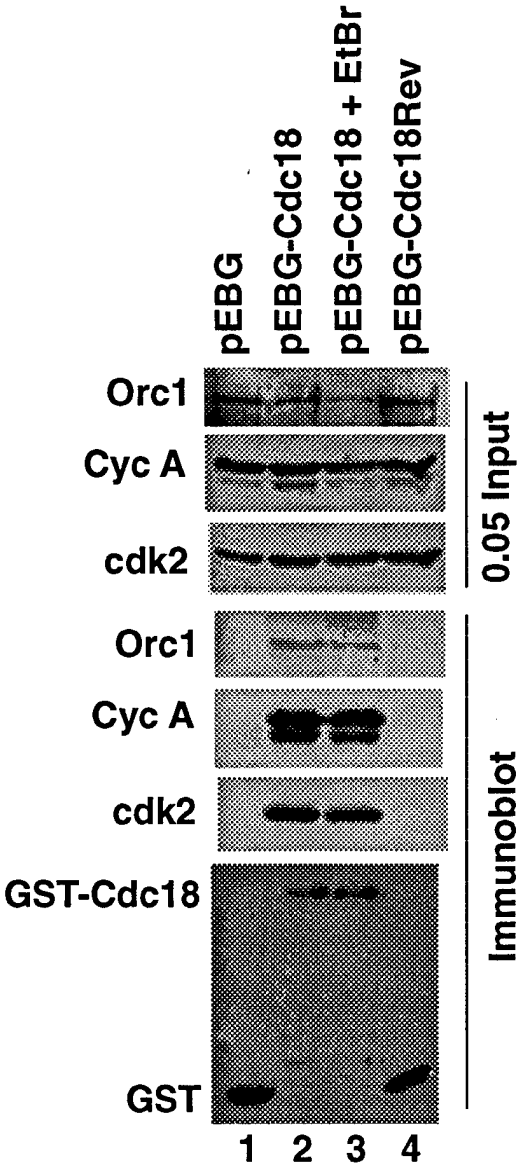
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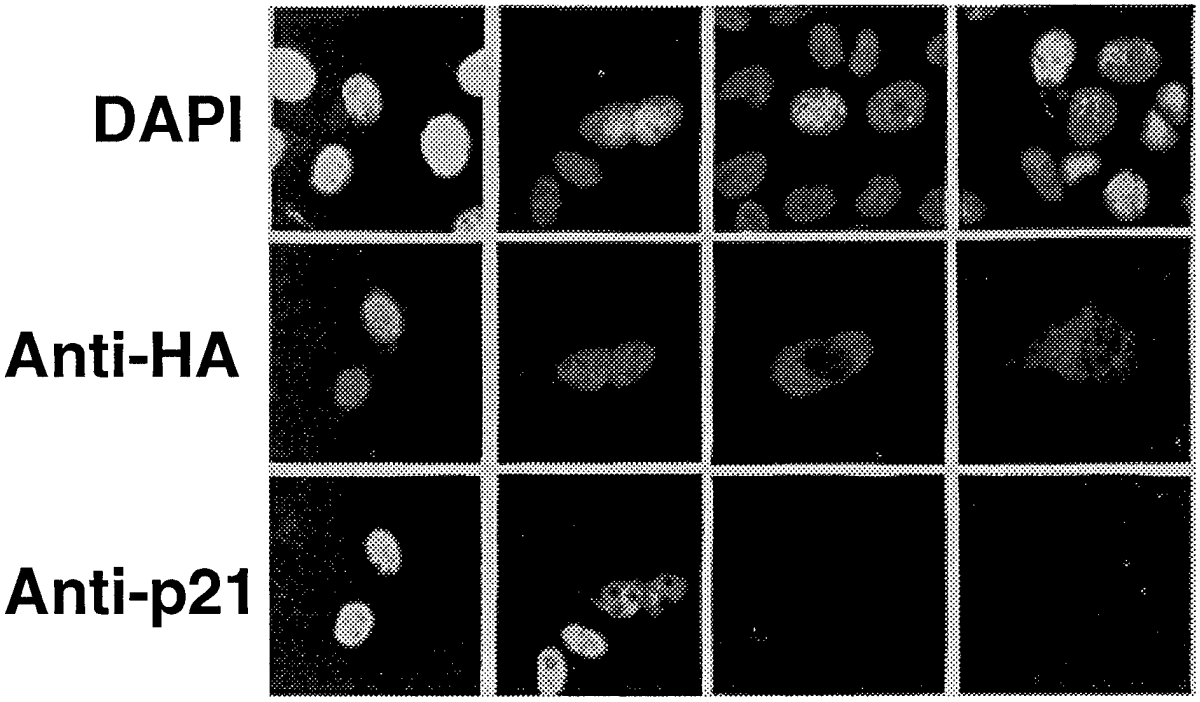
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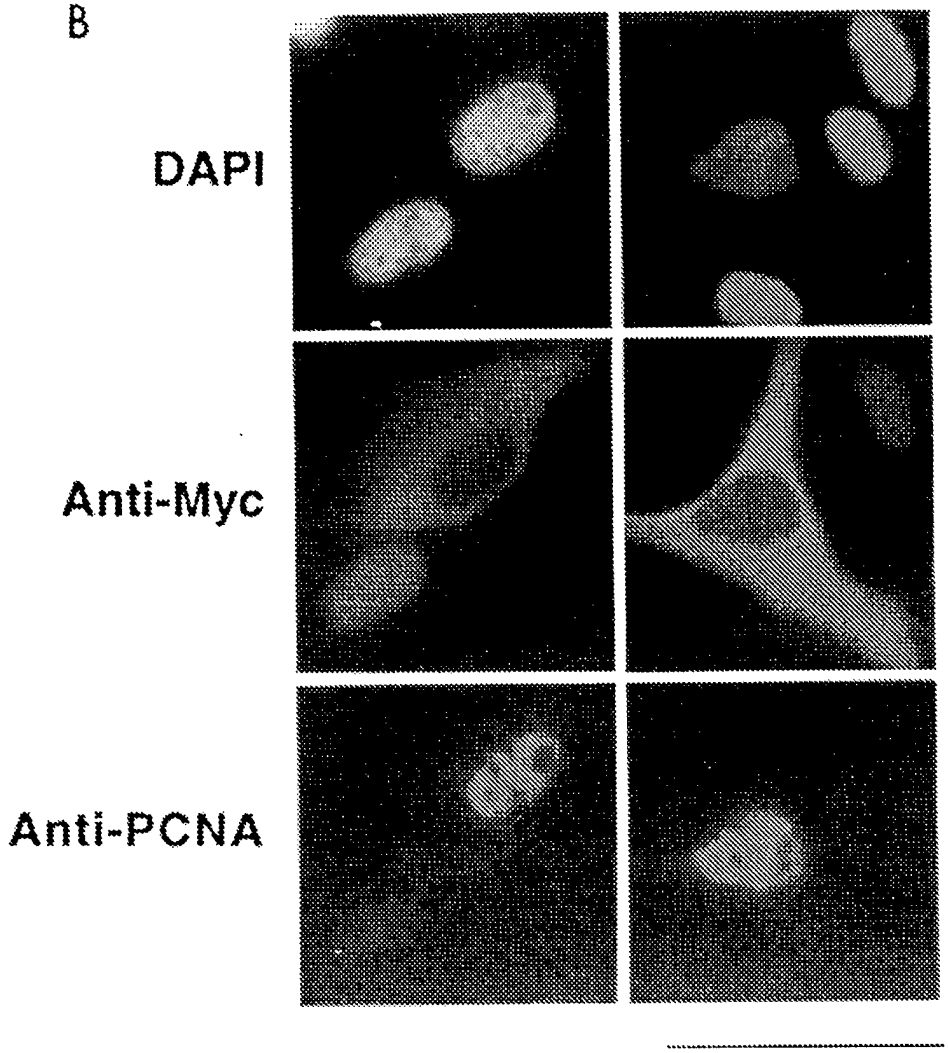
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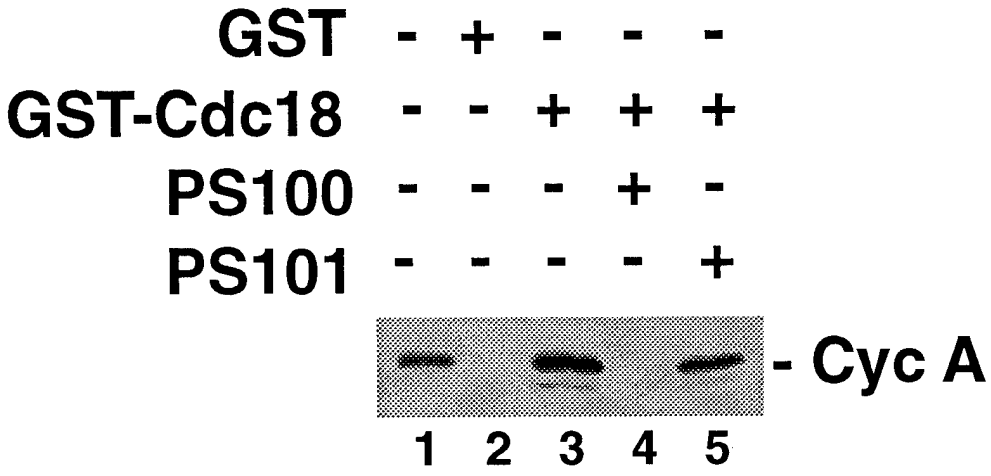
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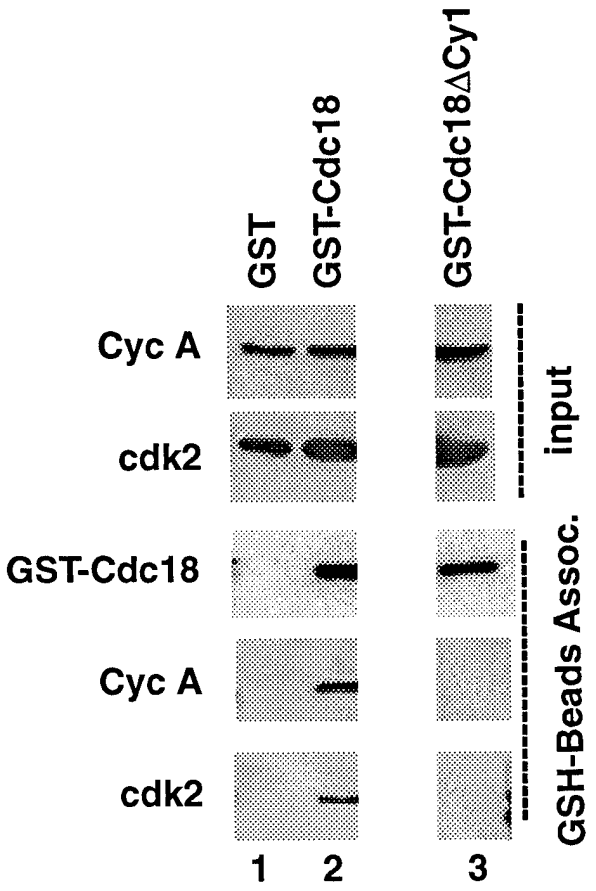
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CONCLUSIONS

Since this is the final report we will summarize here the salient findings from this project over the last 4 years:

1) The interaction of RPA with p53 needs a motif containing amino acids W53 and F54 of p53. When this is mutated, the p53 fails to interact with RPA but can still suppress cell growth. Therefore contrary to original hypothesis p53 does not need to sequester RPA to inhibit cell growth. In contrast, mutations in p53 that affect its transcription activation domain (amino acids L22, W23 of p53), allow it to continue interacting with RPA but severely impair its ability to block the cell-cycle.

2) One of the major transcription targets of p53 is p21, which interacts with and inhibits both cyclin-cdks and the DNA replication factor PCNA. The inhibition of cyclin-cdks by p21 but not the inhibition of PCNA was found to be critical for growth suppression by p53.

3) p21 requires at least one cyclin binding Cy motif (RRLFG) and on cdk binding K motif to interact with and optimally inhibit the cyclin-cdk and stop cell-cycle progression. A Cy peptide can competitively inhibit the interaction between p21 and cyclin-cdks implying that the cyclin-Cy interaction is a docking interaction that has to be in place before the more complicated (and higher affinity) cdk-K interaction can occur.

4) The Cy motif turned out to be important in several substrates of cdks which are known to stably associate with the kinase (e.g. E2F, p107, Rb etc.). We extended the paradigm further by demonstrating that an activator of cdks, Cdc25A, also uses a Cy motif to interact with the cyclin-cdk and that the Cdc25A and p21 molecules can compete for binding to p21. Therefore a cell could up-regulate the levels of several cdk substrates and activators and immunize the cyclin-cdk from inhibition by p21, a situation that might explain why a fraction of breast cancers have high levels of wild type p21 protein and yet continue proliferating.

5) The importance of the Cy motif is highlighted by the discovery in this year's report. A DNA replication factor (Cdc18) was identified as a potential substrate for cdks because of the presence of a functional Cy motif. Mutation of the motif on Cdc18 abolishes stable complex formation between Cdc18 and cyclin-cdks in vivo and prevents the disappearance of nuclear Cdc18 that is normally seen when cyclin-cdks are activated at the G1-S transition.

This grant was only for the salary support of the P.I. Overall it has allowed us to:

explore how a tumor suppressor (p53), commonly mutated in breast cancers, blocks the cell-cycle at the G1-S transition;
discover a pivotal cyclin-binding Cy motif present on inhibitors, substrates and activators of cyclin-cdk;
hypothesize how breast cancer cells could escape from inhibition by up-regulated p21 by up-regulating activators and substrates without mutating the p21 gene and
identify a potential substrate of cyclin-cdks at the G1-S boundary (Cdc18) that is directly involved in the initiation of DNA replication.

A thorough understanding of the regulation of G1-S transition will allow us to determine how breast cancer cells bypass controls that exist at this point in normal cells. Our discovery that up-regulation of molecules like Cdc25A (and other cellular proteins with functional Cy motifs) can displace p21 from cyclin-cdk complexes is an example of such a determination. This result may explain how breast cancer cells paradoxically proliferate despite having up-regulated the levels of p21, a brake that blocks the cell-cycle in normal cells. Cancer cells with wild type p53 respond better to chemotherapy because the DNA damage activates p53 dependent apoptotic pathways. However, a parallel pathway activated by p53 results in p21 mediated G1-S block which protects the cell from apoptosis by giving it time to repair its DNA. Therefore if the p21 block can be bypassed (naturally by up-regulation of Cy containing molecules like Cdc25A and Cdc18: therapeutically by chemicals that trap the p21 in non-functional complexes) then we may potentiate

the DNA damage and apoptotic pathway following chemotherapy and/or radiotherapy in breast cancers with wild type p53.

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MEETING ABSTRACTS

Cold Spring Harbor DNA Replication Meeting, Sept. '95

INTERACTIONS OF P53 AND P21 WITH DNA REPLICATION PROTEINS.

Junjie Chen, Lorene Leiter, Partha Saha, Peter K. Jackson[2], Michele Pagano[3] and Anindya Dutta. Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; [2]Dept. of Cell Biology, Harvard Medical School; [3]Mitotix Inc., Cambridge, MA 02139.

The p53 tumor suppressor is a major focus of current cancer research, because mutations in the p53 gene are the most common genetic alterations in human cancers. Over-expression of p53 arrests cell growth at the G1-S boundary in the cell-cycle. One mechanism by which p53 acts as a growth suppressor is by activating the transcription of a number of genes, e.g. p21. p21 itself interacts with and inhibits two different targets essential for cell-cycle progression. The N terminal half of p21 interacts with and inhibits the cyclin-cdk kinases, while the C terminal half interacts with and inhibits the DNA replication factor PCNA. Using these separated domains, we have determined that p21 inhibits different biological systems through different targets. The PCNA binding domain is sufficient for inhibiting SV40 based DNA replication and for preventing the entry of serum stimulated diploid fibroblasts into S phase. The cdk2 binding domain is sufficient for inhibiting *Xenopus* egg extract based DNA replication and for suppressing growth in transformed human cells and in serum stimulated diploid fibroblasts. The Kd of the p21-PCNA interaction is in the nM range. Although a smaller peptide from p21 could bind PCNA, the interaction was thermodynamically unstable, putting limits on designing small p21 based peptides targetted at PCNA for inhibiting cell growth.

Although p21 is a major downstream effector of p53's growth suppression, p53 also interacts with and inhibits another DNA replication factor, RPA. We have made point-mutations in p53 which affect its ability to bind RPA but not its ability to activate transcription. These mutations do not affect the ability of p53 to act as a growth suppressor in transformed human cells, suggesting that interaction with RPA is not necessary for growth suppression by p53 in this assay.

Cold Spring Harbor Cell Cycle Meeting, May '96

Cyclin-binding motifs are essential for the function of p21/CIP1. Junjie Chen, Partha Saha, Sally Kornbluth, Brian D. Dynlacht and Anindya Dutta. Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

The cyclin-dependent kinase inhibitor p21 is induced by the tumor suppressor p53 and is required for the G1-S block in cells with DNA damage. We report that there are two copies of a cyclin binding motif in p21, Cy1 and Cy2, which interact with the cyclins independent of cdk2. The cyclin binding motifs of p21 are required for optimum inhibition of cyclin/cdk kinases *in vitro* and for growth suppression *in vivo*. Peptides containing only the Cy1 or Cy2 motifs partially inhibit cyclin/cdk kinase activity *in vitro* and DNA replication in Xenopus egg extracts. A monoclonal antibody which recognizes the Cy1 site of p21 specifically disrupts the association of p21 with cyclin E/cdk2 and with cyclin D1/cdk4 in cell extracts. Taken together, these observations suggest that the cyclin binding motif of p21 is important for kinase inhibition and for formation of p21-cyclin/cdk complexes in the cell. Finally, we show that the cyclin/cdk complex is partially active if associated with only the cyclin binding motif of p21, providing an explanation for how p21 is found associated with active cyclin/cdk complexes *in vivo*. The Cy sequences may be general motifs used by cdk inhibitors or substrates to interact with the cyclin in a cyclin/cdk complex.

Cold Spring Harbor DNA Replication Meeting, Sept. '97

HUMAN CDC6/CDC18 ASSOCIATES WITH ORC1, CYCLIN/CDK AND MCM PROTEINS AND IS NOT REGULATED BY CHANGES IN ITS CELLULAR CONCENTRATION THROUGH THE CELL-CYCLE. Partha Saha, Junjie Chen, David Garcia-Quintana, Marvin Hendricks, Kelly C. Thome, Zhi-hui Hou, Jeffrey D. Parvin and Anindya Dutta. Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

In a two-hybrid screen for proteins that interact with human PCNA, we identified and cloned a human protein homologous to yeast CDC6/Cdc18 (HsCdc18). Although the abundance of HsCdc18 transcript increases in S phase, the level of the protein is unchanged throughout the cell-cycle. However, the protein is found variably in nuclear and cytoplasmic compartments, suggesting that cell-cycle regulation of the activity of HsCdc18p may be effected through changes in its sub-cellular localization. In cell extracts, HsCdc18p associates with free HsOrc1p which is not complexed with HsOrc2p, another member of the human origin recognition complex. Thus, unlike in yeasts and Xenopus egg extracts, the interactions between the subunits of the origin recognition complex are less stable in asynchronous cells than the interactions between HsOrc1p and HsCdc18p. The MCM proteins associate directly with HsCdc18p in vitro, consistent with the role of the latter in loading MCM proteins onto chromatin. HsCdc18p also associates with cyclin-dependent kinases through the cyclin component both in vivo and in vitro, providing a mechanism by which the S phase promoting kinase can be recruited to the initiator protein complex. The conserved associations between HsCdc18p and these cell cycle regulatory proteins suggest that HsCdc18p, like its counterparts in yeast and Xenopus, will play an essential role in DNA replication by acting as an adapter between the Origin recognition complex and other proteins essential for the initiation of DNA replication. However, the regulation of these associations through the cell-cycle may differ in detail between species.

Cold Spring Harbor Cell Cycle Meeting, May '98

Human homologs of *Saccharomyces cerevisiae* replication proteins CDC45 and CDC6 Partha Saha, Kelly C. Thome, Zhi-hui Hou, Stephanie Lawlis, James Wohlschlegel and Anindya Dutta Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

In budding yeast *S. cerevisiae* CDC45 is an essential gene required for initiation of DNA replication. A structurally related protein Tsd2 is necessary for DNA replication in *Ustilago maydis*. We have identified and cloned the gene for a human protein homologous to the fungal proteins. Human CDC45L is 30 kB long and contains 15 introns. The 16 exons encode a protein of 566 amino acids. The human protein is 52 and 49.5% similar to CDC45p and Tsd2p, respectively. The level of CDC45L mRNA peaks at G1-S transition, but total protein amount remains constant throughout the cell cycle. Consistent with a role of CDC45L protein in the initiation of DNA replication it co-immunoprecipitates from cell extracts with a putative replication initiator protein, human ORC2L. In addition, sub-cellular fractionation indicates that the association of the protein with the nuclear fraction becomes labile after the initation of DNA replication. The CDC45L gene is located to chromosome 22q11.2 region by cybergenetics and by fluorescence in situ hybridization (FISH). This region, known as DiGeorge syndrome critical region (DGCR), is a minimal area of 2 mB which is consistently deleted in DiGeorge syndrome and related disorders. The syndrome is marked by parathyroid hypoplasia, thymic aplasia or hypoplasia and congenital cardiac abnormalities. CDC45L is the first gene mapped to the DGCR interval whose loss may negatively affect cell proliferation.

CDC6 (from *S. cerevisiae*) or Cdc18 (from *S. pombe*) is a critical component of the pre-replication complex which is proteolytically degraded once DNA replication initiates. The human homolog of CDC6 (CDC6L/Cdc18L) is selectively exported from the nucleus to the cytoplasm at the onset of S phase. Mutations in CDC6L indicate that physical interaction of CDC6Lp with cyclin-cdks is essential for the S phase specific removal of CDC6Lp from the nucleus. Thus as in the yeasts, cyclin-cdks restrict the availability of CDC6Lp once replication has initiated and thereby ensure the alternation of S and M phases in the normal cell-cycle.

The activity of p21, an effector of the tumor suppressor protein p53, in stopping cell-cycle progression. Anindya Dutta. Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

p53, a tumor suppressor gene mutationally inactivated in up to 50% of breast cancers, was postulated to exert its growth suppressive action through two independent mechanisms. The p53 protein is a sequence specific transcriptional activator. It also physically associates with and inactivates a DNA replication factor, RPA. Site directed mutagenesis of the gene encoding the N terminal 70 amino acids of p53 produced forms of p53 which did not bind to RPA but retained wild type transcription activation and forms that had the converse property. Expression of these mutant forms of p53 in transformed human cell lines demonstrated that transcription activation and not RPA inactivation was the major activity required from p53 to suppress cancer cell growth. p21/WAF1/CIP1 is the best candidate target gene whose transcription up-regulation by p53 stops the cell-cycle. In a separate study we noted that about 10% of invasive breast cancers have high levels of expression of p21 (compared to normal mammary epithelium and other breast cancers) and yet continue to proliferate rapidly. Studies by other groups did not find mutations in the p21 gene to account for such a high frequency of p21-resistant breast cancers. p21 was also known to have two separate targets, the cell-cycle regulatory cyclin-dependent kinase (cdk) and the DNA replication factor proliferating cell nuclear antigen (PCNA). To analyze the apparent p21 resistance of the 10% breast cancers, we first determined which of these targets of p21 was critical for cell growth suppression. Appropriate mutations in p21 selectively inactivated the ability of the protein to inhibit cyclin-cdks or PCNA. These mutant forms of p21 indicated that cyclin-cdk inhibition was the critical activity required of p21 to suppress cell growth. Therefore we hypothesized that 10% of breast cancers may have evolved mechanisms by which inhibition of cyclin-cdks by p21 is countered.

To analyze these mechanisms we first determined how p21 inhibits cyclin-cdks. Small mutations placed in the p21 gene demonstrated that the protein uses special sequence elements to bind independently to the catalytic cdk subunit (the K motif) and to the regulatory cyclin subunit (Cy motif). Both these motifs have to be engaged for p21 to stably associate with cyclin-cdks and inhibit the kinase optimally. Although the importance of the interaction of the K motif with the catalytic cdk subunit was well appreciated, the importance of the Cy-cyclin interaction was less clear. 12 amino acid long peptides representing the Cy motif of p21 and a monoclonal antibody to p21 which occupies the Cy sequence of p21 interfered with the stable association of p21 with cyclin-cdks. We inferred that the Cy-cyclin interaction of p21 is of paramount importance for the subsequent K-cdk interaction, so that interference with the Cy-cyclin interaction will diminish the effectiveness of p21 on cyclin-cdks.

Over-expression of cyclins (as noted by us and others in breast cancers) could titrate p21 away from cyclin-cdks and raise the threshold of tolerance of cells to p21. We are currently examining another hypothesis: whether cellular molecules that contain Cy like sequences interfere with the action of p21 on cyclin-cdks. A strong candidate molecule is the cell-cycle activator Cdc25A, which was known to associate with cyclin-cdks stably and promote the G1-S transition by removing inhibitory phosphates from the cdk subunit. Cdc25A has also been reported to be over-expressed in 30-50% of breast cancers and to be a transforming oncogene which co-operates with ras in transformation of primary cells. We demonstrated that Cdc25A has a Cy motif and mutations in this motif inactivate its ability to associate with cyclin-cdks in vivo. There was cross-competition between Cdc25A and p21 for association with cyclin-cdks. High levels of p21 sequester cyclin-cdks away from Cdc25A and prevent the activation of the kinase by Cdc25A. Conversely, cyclin-cdks pre-complexed with Cdc25A have an elevated threshold of tolerance for p21. Thus high concentrations of Cdc25A can protect the cell-cycle from inhibition by moderate levels of p21 which would otherwise have stopped the cell-cycle progression. We postulate that Cdc25A and similar cellular molecules which associate stably with cyclins through Cy-like motifs are up-regulated in breast cancers and are partially responsible for the apparent p21-resistance of 10% of breast cancers.

In a parallel line of enquiry we sought to develop potentially therapeutic molecules that can suppress cancer cell growth by inhibiting PCNA. Although the critical target of physiological levels of p21 in cells is cyclin-cdk, high concentrations of p21 can interfere with the action of

PCNA and suppress cell growth independent of any action on cyclin-cdk. Small chemicals that mimic the action of p21 on PCNA could be novel pharmacological agents against breast cancer. A 39 amino acid peptide from the C terminus of p21 indeed interferes with the action of PCNA in vitro. The p21-PCNA interaction has been noted to inhibit the action of PCNA as a processivity factor for DNA polymerase delta, a key enzyme essential for the replication of DNA during cancer cell proliferation. We discovered that PCNA independently associates with a 5'-3' exonuclease, Fen1, which is required for complete DNA replication and repair. Further, p21 and the 39 amino acid fragment of p21 which interacts with PCNA both disrupt the Fen1-PCNA association. These studies suggest that p21-mimetic chemicals interacting with PCNA could potently inhibit DNA replication and cell growth by disrupting multiple protein-protein interactions between replication enzymes and the PCNA scaffold that tethers these enzymes to the replication fork.

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Human CDC6/Cdc18 Associates with Orc1 and Cyclin-cdk and Is Selectively Eliminated from the Nucleus at the Onset of S Phase

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In a two-hybrid screen for proteins that interact with human PCNA, we identified and cloned a human protein (hCdc18) homologous to yeast CDC6/Cdc18 and human Orc1. Unlike yeast, in which the rapid and total destruction of CDC6/Cdc18 protein in S phase is a central feature of DNA replication, the total level of the human protein is unchanged throughout the cell cycle. Epitope-tagged protein is nuclear in G_1 and cytoplasmic in S-phase cells, suggesting that DNA replication may be regulated by either the translocation of this protein between the nucleus and the cytoplasm or the selective degradation of the protein in the nucleus. Mutation of the only nuclear localization signal of this protein does not alter its nuclear localization, implying that the protein is translocated to the nucleus through its association with other nuclear proteins. Rapid elimination of the nuclear pool of this protein after the onset of DNA replication and its association with human Orc1 protein and cyclin-cdks supports its identification as human CDC6/Cdc18 protein.

The recent identification of multiple eukaryotic proteins that bind directly or indirectly to origins of DNA replication has set the stage for a thorough exploration of how DNA replication is initiated and regulated in eukaryotes. Central to the process is the origin recognition complex (ORC), a tight complex of six polypeptides identified initially in *Saccharomyces cerevisiae* because it binds in a sequence-specific manner to origins of DNA replication and is involved in the initiation of chromosomal DNA replication (1, 3, 27). Homologs of three of the subunits of the ORC (Orc1, Orc2, and Orc4) have been identified in humans (16, 32) and in other eukaryotes (14, 17, 22, 28).

An additional molecule, CDC6 in *S. cerevisiae* and Cdc18 in *Schizosaccharomyces pombe*, is essential for the onset of DNA replication and known to associate physically with the ORC and cdc2 kinase (2, 12, 15, 18, 22, 24, 37). The yeast CDC6/Cdc18 proteins decrease in concentration as cells proceed through S phase, with overexpression of Cdc18 in *S. pombe* resulting in the rereplication of DNA in G_2 (29). The *Xenopus* CDC6/Cdc18 and ORC are required for DNA replication and for the loading of the minichromosome maintenance (MCM) proteins onto the chromatin (6, 11, 13, 19, 34, 35). Collectively, a model has emerged that emphasizes the central role of CDC6/Cdc18 in cooperating with ORC and MCM proteins to form a prereplication complex at origins of DNA replication in G_1. Once replication begins, the concordant removal of CDC6/Cdc18 prevents the loading of MCM proteins onto the origin-bound ORC in G_2, thereby preventing rereplication of DNA. After mitosis, synthesis of new CDC6/Cdc18 protein allows the loading of MCM proteins on the chromatin, perhaps by forming a bridge between the chromatin-bound ORC and the MCM proteins (4). The prereplication initiation complex thus formed in G_1 is ready to initiate DNA replication upon the activation of S-phase-promoting factors like cyclin-cdk's and CDC7 kinase.

We identified and cloned a human protein that is structurally homologous to yeast CDC6/Cdc18 and Orc1 proteins by virtue of its interaction with human PCNA in a two-hybrid assay. A similar human protein was independently identified by Williams and coworkers because of its sequence homology with Orc1 and CDC6/Cdc18 and was named human CDC6 protein (36). Biochemical experiments with this protein reported here confirm that it is the putative human CDC6/Cdc18 but also indicate that, although the overall model of replication regulation is conserved between humans and yeasts, some aspects are different in detail.

MATERIALS AND METHODS

Yeast two-hybrid screen. For the yeast two-hybrid screen, the open reading frame encoding human PCNA was cloned by PCR and inserted into the pAS2 vector to create a fusion protein with the GAL4 DNA-binding domain (7). The interacting plasmid from a B-cell cDNA library (human PCNA interacting protein 4; HPB4) had a 1.2-kb cDNA insertion encoding a protein fragment whose sequence was closely related to Cdc18 from *S. pombe* and to human Orc1 (hOrc1).

Cloning of hCdc18 gene. Using HPB4 as a probe, we isolated a 1.6-kb cDNA from a human fetal brain cDNA library. This 1.6-kb cDNA overlapped with the HPB4 gene and extended the 5′ terminus by 600 bp. The extreme 5′ terminus of the human Cdc18 (hCdc18) gene was found by employing 5′ rapid amplification of cDNA ends (RACE) with human HeLa Marathon-Ready cDNA (Clontech), which extended the 5′ end of the Cdc18 clone by 300 bases. There is an in-frame stop codon 150 bp 5′ of the first methionine. The 3′ end of the hCdc18 gene cDNA came from the expressed sequence tag (EST) clone T85849 (GenBank), which had a 150-base overlap with the 3′ end of the HPB4 gene sequence, and extends the nucleotide sequence by 1,000 bases and the protein sequence by five amino acids. The hCdc18 gene cDNA was constructed by combining the RACE PCR product, HPB4 gene insert, and T85849 EST clone and contains an open reading frame flanked at both ends by stop codons. The predicted protein consists of 560 amino acids with a calculated molecular mass of 62.7 kDa.

RNA analysis. Total RNA was extracted from HeLa cells as described previously (10), and 10 μg was subjected to Northern blot analysis at 42°C. The probes used were a 1.2-kb cDNA fragment from the HPB4 gene (nucleotides 520 to 1750 of the hCdc18 gene), a 2.4-kb cDNA fragment from pKG28 (the entire open reading frame encoding hOrc1 [16]), a 1.3-kb *Hin*dIII-*Pst*I cDNA fragment (encoding glyceraldehyde-3-phosphate dehydrogenase), and cDNA fragments

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containing the entire open reading frames encoding cyclins E, A, B, and hOrc2 (16).

Plasmid construction and protein purification. Full-length hCdc18 (amino acids 1 to 560) and its fragments (amino acids 1 to 179, 179 to 407, and 407 to 560) were generated by PCR with appropriate pairs of oligonucleotide primers and *Pfu* polymerase (Stratagene) and cloned into *Bam*HI and *Sal*I sites of pGEX vector (Pharmacia) to generate glutathione *S*-transferase (GST) fusion proteins (9).

The full-length hCdc18 was cloned into mammalian expression vectors pA3M and pAHP to generate plasmids expressing hCdc18 with a myc (pA3M) epitope tag at the C terminus or hemagglutinin (pAHP) epitope tag at the N terminus. Plasmid pEBG expresses GST in mammalian cells (26). The coding region of the Cdc18 gene was cloned into the *Bam*HI site of pEBG to generate pEBG-Cdc18 (sense orientation) and pEBG-Cdc18rev (antisense orientation). The former expresses GST-Cdc18 (90 kDa), and the latter expresses GST fused to a short irrelevant protein expressed from the antisense strand of the Cdc18 gene (30 kDa).

Cell synchronization. Exponentially growing HeLa or U2OS cells were arrested for 24 h with 10 mM hydroxyurea at early S phase, with 40 ng of nocodazole/ml at M phase, or with 5 μg of aphidicholin/ml at the G_1-S transition point. The cell populations were checked by propidium iodide staining of the DNA and by flow cytometry. For serum starvation and release experiments with NIH 3T3, CV1, and WI38 cells, 60%-confluent cultures were placed in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% serum for 48 h and split 1:2 into DMEM with 10% calf serum.

Pull-down assay. Affinity chromatography on glutathione beads coated with various GST fusion proteins (pull-down assay) was done as described previously (8, 9). Incubation of MCM proteins with GST- or GST-Cdc18-coated beads was carried out at 37°C for 10 min followed by 4°C for 1 h. The unbound proteins were washed off with A7.4 buffer containing 100 mM NaCl.

Antibodies and immunoprecipitations. Antibody against hCdc18 was raised against a GST protein fused to a fragment of hCdc18 protein from amino acids 145 to 326. The antibody was affinity purified on the antigen to confirm the immunoblot experiments. Antibody against hOrc1 was raised against a recombinant His_6-tagged fragment of Orc1 from amino acids 647 to 861 created by cloning the 1.1-kb *Eco*RI-*Hin*dIII fragment of EST clone 121313 (GenBank) into pRSETB (Invitrogen). Antibody against hOrc2 was raised against a recombinant His_6-tagged fragment of Orc2 from amino acids 27 to 577 created by cloning the *Xba*I-*Sac*I Orc2 fragment into the *Pvu*II site of pRSETC. Immunoblotting of cell lysates (20 μg of protein) was carried out with the antibodies at the following dilutions: anti-hCdc18, 1:5,000; anti-hOrc1, 1:15,000; anti-hOrc2, 1:2,000; and 9E10 anti-Myc monoclonal antibody ascites, 1:2,000.

Immunofluorescence. U2OS cells were transfected with pAHP-Cdc18 or with pA3M-Cdc18. The cells were fixed in 3% formaldehyde containing 2% sucrose at room temperature for 10 min, washed twice with phosphate-buffered saline (PBS), and permeabilized in Triton solution (3% bovine serum albumin, 0.5% Triton X-100 in PBS) for 5 min. After being washed with PBS, the cells were incubated for 20 min at 37°C with anti-hemagglutinin (HA) antibody (12CA5) in hybridoma culture supernatant diluted 1:100, anti-Myc monoclonal antibody in mouse ascites diluted 1:100, anti-p21 rabbit polyclonal antibody (Santa Cruz) diluted 1:100, or human autoimmune antibody to PCNA. Cells were then washed three times with PBS and incubated with the secondary antibody (fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse immunoglobulin G [IgG], rhodamine-conjugated goat anti-rabbit IgG, or anti-human IgG [Jackson Laboratory]). After being washed three times with PBS, the cells were stained briefly with DAPI (4′,6-diamidino-2-phenylindole) and photographed under a fluorescent microscope.

Preparation of HeLa whole-cell extract and column chromatography. HeLa whole-cell extract was prepared according to the procedure of Manley et al. (25). All steps were performed at 4°C. Briefly, a HeLa cell pellet was suspended and incubated on ice for 20 min in 4 volumes of buffer WCE I (10 mM Tris-acetate [pH 7.9], 1 mM EDTA, 5 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were lysed in a Dounce homogenizer with eight strokes and then mixed with another 4 volumes of buffer WCE II (50 mM Tris acetate [pH 7.9], 10 mM Mg acetate, 25% sucrose, 50% glycerol, 2 mM DTT, and 1 mM PMSF). Ammonium sulfate was added to a final concentration of 0.4 M, and after stirring for 30 min, the extract was centrifuged at 40,000 rpm in a Ti45 rotor (Beckman) for 3 h. The supernatant was subjected to 65% ammonium sulfate precipitation, after which the pellet was suspended in 20 mM Tris acetate (pH 7.9) containing 1 mM EDTA, 0.15 M potassium acetate, 20% glycerol, 2 mM DTT, and 1 mM PMSF (buffer H), and the suspension was dialyzed against the same buffer. Starting from 3×10^{10} cells, 3.0 g of protein was recovered. The protein extract was applied to a Bio-Rex 70 (Bio-Rad) column (bed volume, 65 ml) equilibrated in buffer H, and the bound proteins were eluted with 0.3, 0.6, and 1.5 M potassium acetate in buffer H. The 0.3 M fraction containing Orc1 and Cdc18 (total protein, 280 mg) was dialyzed and further fractionated over a DEAE-cellulose column (bed volume, 25 ml) equilibrated in buffer H containing 0.2 M potassium acetate. The proteins were eluted with 0.35 and 1.0 M potassium acetate in buffer H. A portion of 0.2 M flowthrough (total protein recovery, 88 mg) containing 3.5 mg of protein was subjected to Mono-S HR 5/5 fast protein liquid chromatography (Pharmacia) column in 0.2 M KCl containing buffer and eluted with a gradient of 0.2 to 0.6 M KCl containing buffer. The flowthrough containing Orc1 and Cdc18 was further characterized by Superose 12 gel filtration chromatography.

RESULTS

Cloning and sequence analysis of hCdc18. While screening for proteins which interact with the human DNA replication factor PCNA in a two-hybrid assay, we discovered an interacting molecule whose sequence was related to *S. pombe* Cdc18 (33% identical, 53% similar), *S. cerevisiae* CDC6 (32% identical, 54% similar), and hOrc1 (28% identical and 52% similar) (Fig. 1). The theoretical mass of the protein is similar to that of yeast CDC6/Cdc18 (60 kDa) and different from that of the Orc1 proteins (100 to 120 kDa). The homology with the Orc1-Cdc18 family is most marked in the middle one-third of the protein, which contains a nucleotide binding motif with the putative P and A loops (20) together with four other conserved boxes unique to this family of proteins (2, 16). The N-terminal one-third has four potential sites of phosphorylation by cyclin-cdk's (serines at positions 45, 54, 74, and 106) and a putative bipartite nuclear localization sequence (NLS) at positions 80 to 95 (33) (Fig. 1B).

As shown in Fig. 1, the sequence homology of the newly identified protein with hOrc1 leaves open the possibility that it is a member of the Orc1-Cdc18 family and is not necessarily the human CDC6/Cdc18. We have independently identified a hOrc4 which, surprisingly, has sequence similarity to the Orc1-Cdc18 family of proteins, implying that there may be an entire family of human proteins with sequence homology to yeast Orc1 or Cdc18 (32). The protein-protein association and the cell cycle regulation of the new protein reported below, however, provides strong evidence that it is indeed human CDC6/Cdc18.

Although hCdc18 transcripts are increased at the G_1-S transition, total Cdc18 protein levels are uniform throughout the cell cycle. One of the characteristic properties of yeast CDC6/Cdc18 is that the highest levels of the mRNA and protein are obtained in the G_1 phase of the cell cycle, with almost all RNA and protein disappearing in the S and G_2 phases. Northern blot analysis of total RNA from HeLa cells at various stages in the cell cycle indicates that with hCdc18, the mRNA level peaks at the onset of S phase (simultaneous with the expression of cyclin E) and diminishes at the onset of mitosis (when cyclin B is expressed) (Fig. 2A). This pattern is repeated for hOrc1 but not hOrc2. Consistent with this observation, cells arrested with hydroxyurea (in S phase) have significantly more Cdc18, Orc1, and cyclin E mRNA than cells arrested with nocodazole (in M phase) (Fig. 2B). Such variation of mammalian Orc1 and Cdc18 mRNA during the cell cycle agrees with observations in the yeasts. The cell cycle stage-specific regulation of mammalian Orc1 and mammalian CDC6/Cdc18 mRNA was reported by others to be due to the activity of E2F transcription factor and was postulated to be important for linking the initiation of S phase with activation of E2F (30, 36).

Since cell cycle stage-specific regulation of mRNA does not always produce an equivalent cell cycle stage-specific regulation of the protein, we examined whether the abundance of the mammalian Orc1 and Cdc18 proteins changed during the cell cycle. An antibody raised against recombinant Cdc18 and immunoaffinity purified recognized a protein of 66 kDa in HeLa cell extracts, while one raised against Orc1 recognized a protein of 100 kDa (Fig. 2B). The anti-Cdc18 antibody also recognized a myc epitope-tagged Cdc18 expressed by transient transfection in human cells (data not shown). Although the mRNA levels of Cdc18 and Orc1 were much higher in S-phase (hydroxyurea-blocked) than in M-phase (nocodazole-blocked)

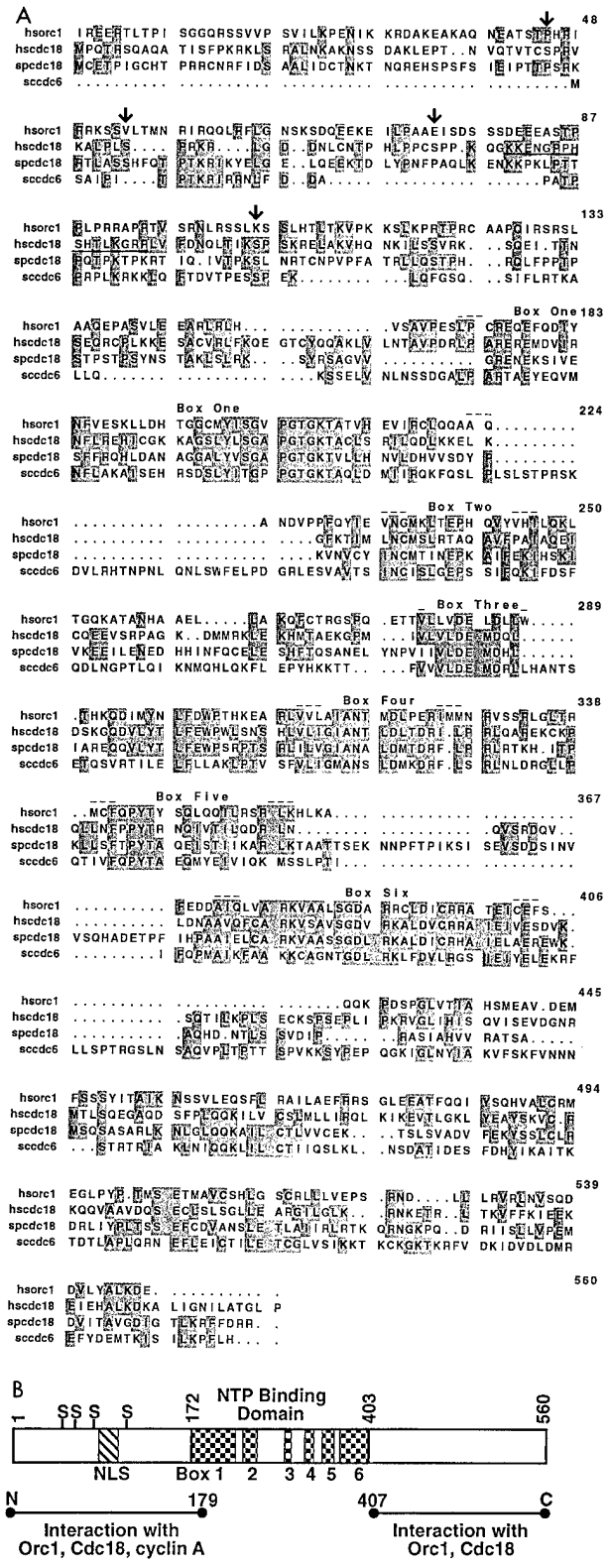

FIG. 1. (A) Alignment of protein sequences of hCdc18 (hscdc18) with those of hOrc1 (hsorc1), *S. pombe* Cdc18 (spcdc18), and *S. cerevisiae* CDC6 (sccdc6). The numbers refer to the sequence of hCdc18. The alignment was done with the PILEUP program of the Genetics Computer Group package. Identical amino acids are shaded. Arrows mark putative substrate sites for cyclin-cdk's (on the hscdc18 protein), and the putative bipartite NLS is underlined. Conserved boxes 1 to 6 are indicated; boxes 1 and 3 contain the P and A loops, respectively. (B) Schematic representation of the hCdc18 protein divided into three domains. Boxes 1 to 6 in the middle one-third of the protein are the same as in panel A. S, a serine in a putative cyclin-cdk phosphorylation site; NLS, putative bipartite nuclear localization sequence; NTP, nucleoside triphosphate. The horizontal lines represent recombinant fragments of Cdc18 which are sufficient to mediate the interactions with Orc1, Cdc18 (Fig. 4A), and cyclin A (data not shown).

cells, the protein levels were virtually unchanged between the two phases of the cell cycle. To ensure that the constant levels of total cellular Cdc18 protein was not an anomaly of transformed HeLa cells, we serum starved untransformed mouse NIH 3T3 cells and followed CDC6/Cdc18 protein levels after serum stimulation (Fig. 2C). The serum-starved cells had a significant amount of Cdc18 protein, with little change in the level of the protein as the cells moved from G_0 through G_1 into the S phase of the cell cycle upon release from serum starvation (Fig. 2C). The band of slightly reduced mobility seen in G_1 is weaker but present in G_0 cells and could be due to post-translational modification of Cdc18 protein as cells enter the cell cycle. Both bands are recognized by affinity-purified anti-Cdc18 antibody. The levels of Orc1 and Orc2 proteins are similarly unchanged in this experiment. The constant levels of Orc2 and PCNA proteins demonstrate that all lanes contain protein from equivalent numbers of cells. The transient appearance of cyclin A shows that the proteins which are expected to cycle as cells passage through G_1 into S do so normally in this experiment. Similar results were obtained with untransformed monkey CV-1 and primary human WI38 cells (data not shown). Therefore, the G_1-S peak in the expression of Cdc18 or Orc1 mRNAs is not paralleled by changes in the total protein concentration.

Subcellular localization of hCdc18 protein changes during cell cycle. The previous report on human CDC6/Cdc18 (36) noted that the protein associated with the nuclear fraction was transiently increased in G_1 and early S and decreased thereafter. The authors concluded that the human CDC6/Cdc18 protein is regulated by cell cycle stage-specific degradation similar to that noted in the yeasts. Since we did not observe a change in the abundance of total cellular protein throughout the cell cycle, one explanation could be that the subcellular localization of CDC6/Cdc18 protein changed during the cell cycle, with increased nuclear protein seen in G_1 and early S. To test this possibility, the subcellular localization of Cdc18 protein was determined by immunofluorescence.

Because the anti-Cdc18 antibody did not immunostain cells, we expressed Cdc18 with an N-terminal HA epitope tag by transient transfection of human U2OS osteosarcoma cells and immunostained the cells with anti-HA antibody (12CA5). The results were confirmed with Cdc18 fused to a C-terminal myc epitope. Of 100 asynchronous cells expressing epitope-tagged Cdc18, 20 to 40% expressed the protein in the cytoplasm and 60 to 80% expressed it in the nucleus in different experiments (Fig. 3A and B). Thirty percent of the cells were in G_1, as judged by positive staining for nuclear p21 (CIP1) (23). In all of these G_1 cells, HA-hCdc18 protein was detected in the nucleus (Fig. 3A). All cells where HA-hCdc18 protein was found in the cytoplasm were negative for p21 (Fig. 3A), implying that HA-hCdc18 protein was seen in the cytoplasm in some phase other than G_1.

To further clarify when during the cell cycle hCdc18 protein appears in the cytoplasm, we expressed myc epitope-tagged hCdc18 protein in U2OS cells and stained the cells with anti-myc and anti-PCNA antibodies (Fig. 3B). Among the 120 cells expressing Myc-hCdc18 protein, 30 to 40% were in S phase, as judged by nuclear staining for PCNA (23). Myc-hCdc18 pro-

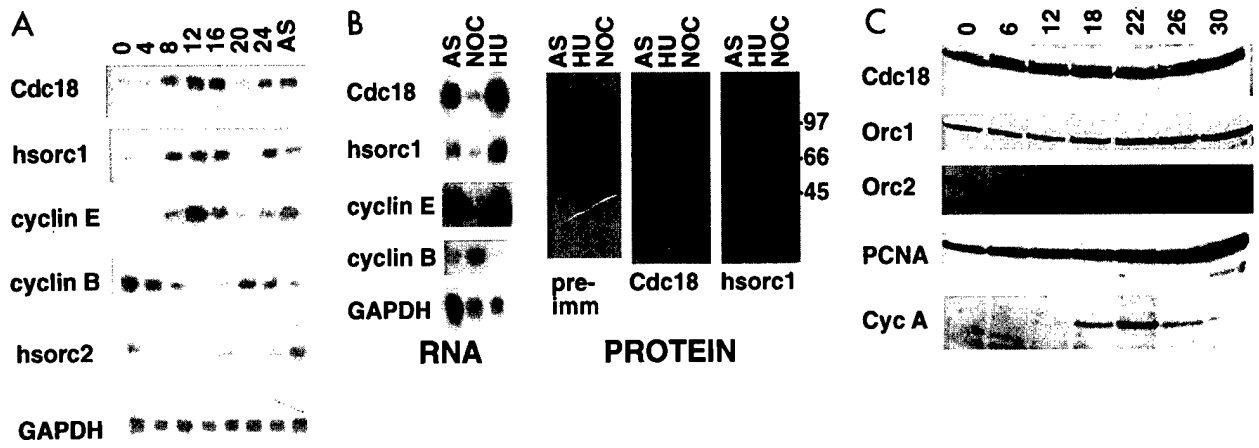

FIG. 2. Levels of Cdc18 and Orc RNAs and proteins in different stages of the cell cycle. (A) HeLa cells were synchronized in mitosis by nocodazole (50 ng/ml), and after shake off, the cells were replated in nocodazole-free medium. A Northern blot of RNA extracted from the cells at the indicated number of hours after release is shown. AS, asynchronous cells; hsorc1 and -2, hOrc1 and -2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) HeLa cells were either asynchronous (AS), blocked in S phase with hydroxyurea (HU), or blocked in mitosis with nocodazole (NOC). RNA, Northern blot of total RNA with the indicated probes; PROTEIN, immunoblots of protein extracts with preimmune serum (pre-imm) (for anti-Cdc18 antibody; the same result was obtained with the preimmune serum for anti-Orc1 antibody or rabbit serum against bacterially produced GST-Cdc18 protein (Cdc18) or the His_6-tagged C-terminal one-third of hOrc1 protein (hsorc1). (C) NIH 3T3 cells were released from serum starvation and harvested at indicated time-points (h) as the cells moved from G_0 through G_1 to S. Extracts were immunoblotted for the indicated proteins. Cyc A, cyclin A.

tein was detected in the cytoplasm of all these cells (Fig. 3B). Whenever myc-Cdc18 protein was detected in the nucleus, the cells were negative for PCNA and were therefore not in S phase. Therefore, hCdc18 protein is present in the nucleus during G_1 phase (p21 positive) in time to form prereplication initiation complexes. After origins have fired in S phase (PCNA positive), hCdc18 protein is seen only in the cytoplasm, reappearing in the nucleus some time before the next G_1.

The above observations were confirmed by immunofluorescence of HA-hCdc18 protein after transient transfection and cell cycle synchronization of U2OS cells (Fig. 3C). In exponentially growing culture, HA-hCdc18 protein was detected in the nuclei of 50% of the cells expressing the protein. When the cells were arrested at G_1-S transition with aphidicholin, HA-hCdc18 protein was detected in the nuclei of 90% of the transfected cells, whereas the protein was detected in the cytoplasm of 80% of the transfected cells arrested at S phase with hydroxyurea.

Thus, although the total amount of hCdc18 protein is unchanged during the cell cycle, the activity is likely to be regulated by changes in its subcellular localization. The increase in nuclear protein in G_1 makes this protein similar to yeast CDC6/Cdc18 rather than yeast Orc1, whose levels are unchanged throughout the cell cycle.

The putative NLS of CDC6/Cdc18 protein is not necessary for nuclear localization. $KK(X)_{10}KGRR$ matches a consensus bipartite (NLS) near the N terminus of CDC6/Cdc18 protein and is conserved in *Xenopus* CDC6 protein (Fig. 1) (13, 33). Because epitope-tagged hCdc18 protein was easily detected in the nucleus, we tested whether its localization was dependent on this putative NLS by mutating the sequence to $KK(X)_{10}KGGG$ and expressing the protein with an N-terminal HA epitope tag (Fig. 3D). The mutant protein was still detected in the nucleus, implying that the bipartite NLS of CDC6/Cdc18 is not required for nuclear localization of Cdc18 protein. This result also suggests that the nuclear localization of Cdc18 protein could be mediated by its association with other cellular proteins that have their own NLSs.

hCdc18 protein associates with free Orc1 protein. Further similarity with yeast CDC6/Cdc18 was noted upon examination of the association of human Cdc18 with other components in the replication initiation complex. Yeast CDC6/Cdc18 interacts with the yeast ORC. GST-hCdc18 protein associated well with hOrc1 protein produced by in vitro transcription-translation (Fig. 4A). Studies with the deletion mutants of hCdc18 revealed that either the N- or C-terminal one-third of the protein could independently associate with Orc1 protein. The middle one-third of the protein, containing the domain with maximum homology to Orc1, was incapable of mediating any of these associations. Although homo-oligomerization of CDC6/Cdc18 protein has not been reported in any species, we observed very robust association between GST-Cdc18 protein and S^{35}-labeled Cdc18 protein produced by in vitro transcription-translation. Consistent with the sequence similarities between Cdc18 protein and Orc1 protein, the same regions of Cdc18 protein were sufficient to interact with Cdc18 protein as with Orc1 protein. Despite the discovery of human CDC6/Cdc18 by a two-hybrid screen with PCNA, neither radiolabeled PCNA produced by in vitro transcription-translation nor recombinant purified PCNA produced in bacteria associated with GST-Cdc18, suggesting that the association in the two-hybrid assay may be mediated by unidentified yeast proteins.

Although Cdc18 and Orc1 proteins associated with each other in vitro, we did not know what fractions of the cellular Cdc18 and Orc1 proteins were physically present in the same complex. To determine this, HeLa whole-cell extract (made with 0.4 M ammonium sulfate) (25) was fractionated over Bio-Rex-70, DEAE-Sepharose, and Mono-S columns. The bulk of Cdc18 protein in the cell extracts cofractionated with Orc1 protein over the three columns (Fig. 4B). Only 15% of the input protein flows through both DEAE-dextran (binds to negative charge) and Mono-S (binds to positive charge) columns as Cdc18 and Orc1 proteins do. The purification of the Cdc18-Orc1 proteins on column 1 is 14-fold, on column 2 it is 1.75-fold, and on column 3 it is 3.7-fold, for a total purification of 90-fold.

To examine whether the coelution of Cdc18 and Orc1 proteins over three columns was due to their presence in the same physical complex, the fraction from the Mono-S column was subjected to gel filtration. Cdc18 and Orc1 proteins coeluted

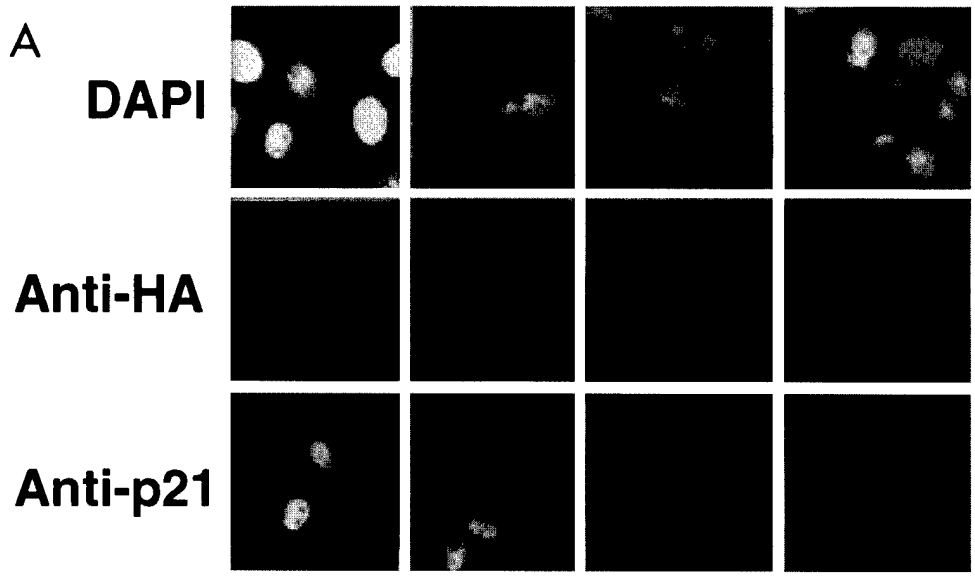

FIG. 3. Subcellular localization of epitope-tagged hCdc18 protein. (A) Nuclear hCdc18 protein is seen in G_1 cells that are positive for nuclear p21. Asynchronously growing human osteosarcoma U2OS cells were transfected with plasmid expressing HA-Cdc18 protein, and 2 days after transfection the cells were stained with DAPI (top row), anti-HA monoclonal antibody (12CA5) and FITC-conjugated goat anti-mouse secondary antibody (middle row), and anti-p21 (CIP1) rabbit polyclonal antibody and rhodamine-conjugated goat anti-rabbit secondary antibody (bottom row). The two columns on the left show positive nuclear staining for p21 (G_1 cells); the two columns on the right show negative staining for p21 (non-G_1 cells). Similar results were obtained when the cells were transfected with the plasmid expressing myc-Cdc18 protein. (B) S-phase cells marked by nuclear PCNA have Cdc18 protein in the cytoplasm. Asynchronously growing U2OS cells were transfected with plasmid expressing myc-Cdc18 protein, and the cells were stained with DAPI (top row), anti-myc monoclonal antibody and FITC-conjugated secondary antibody (middle row), and autoimmune human serum containing anti-PCNA antibody and rhodamine-conjugated secondary antibody (bottom row). (C) Subcellular localization of epitope-tagged hCdc18 protein in synchronized cells. U2OS cells were transfected with plasmid expressing HA-Cdc18 protein and then either grown exponentially (exp) or arrested 24 h posttransfection with aphidicholin (aph) or hydroxyurea (hu). HA-Cdc18 protein was detected by immunofluorescence, and the cells containing HA-hCdc18 in the cytoplasm (C) or the nucleus (N) were counted. The results represent the averages of four different experiments with independent transfections. (D) HA-Cdc18 protein with the NLS mutated is still localized to the nucleus. Details are the same as for the top and middle rows of panel A.

on a Superose 12 (Pharmacia) column in 50 mM KCl, with Orc1 protein eluting as an apparent monomer of about 100 kDa (about one-half to one fraction lighter than where it coelutes with Cdc18 protein in 50 mM KCl). Cdc18 protein elutes as a multimer of 300 kDa when separated from Orc1 protein, consistent with the same region(s) of the protein being utilized for association with itself as with Orc1 protein.

Another component of the human ORC, the 72-kDa hOrc2 protein, separated from the Orc1-Cdc18 complex in the first column, implying that most of the hOrc1 protein and hCdc18 protein was readily separated from other components of the putative six-subunit human ORC (Fig. 4B). The small size of the Cdc18-Orc1 protein complex (120 to 150 kDa) on the gel filtration column also suggests that Cdc18 protein (66 kDa) is associated only with free Orc1 protein (100 kDa) rather than with the entire six-subunit ORC (expected to be greater than 300 kDa). To confirm this, the fractions from the Mono-S column were purified further by sequential chromatography over Mono-Q, Affigel Blue, and Superose 12 columns to obtain Orc1 protein purified to homogeneity (Fig. 4D). This form of Orc1 protein was still capable of associating with GST-Cdc18 protein (data not shown), indicating that Cdc18 and Orc1 proteins associate directly with each other.

Thus, contrary to the situation in the yeasts or *Xenopus* egg extract, the human ORC appears to dissociate under the conditions of cell lysis and chromatography used here. It is interesting that the Cdc18-Orc1 protein association appears to be more stable than the association of Orc1 protein with other ORC subunits.

Association of hCdc18 protein with cyclin-cdk2 and its inhibition by a factor present in mitotic cell extracts. Since Cdc18 protein and cdc2 associate with the ORC in *S. pombe* cells (22) and a direct interaction has also been reported between *S. cerevisiae* CDC6 and CDC28 (15, 31), we tested whether human Cdc18 protein associated with any of the cyclin-cdk's. Cyclins D1, D2, D3, E, A, and B produced by in vitro transcription-translation in a rabbit reticulocyte lysate associated well with GST-Cdc18 protein produced in bacteria (Fig. 5A; compare interaction of cyclins with the positive control, p21). All interactions were stable in the presence of 50 μg of ethidium bromide/ml, showing that the associations are not mediated through nucleic acids (21). cdk2 produced by in vitro transcription-translation did not form a complex with rabbit cyclins (9) and did not associate with bacterially produced GST-Cdc18 protein (Fig. 5A). cdk2 from S-phase extracts of HeLa cells associated with GST-Cdc18 protein (Fig. 5B, lane 2), presumably because of the associated cellular cyclins. The cyclin-cdk's phosphorylated the N-terminal one-third of Cdc18 protein in vitro (data not shown).

Since a mitotic inhibitor of the association of CDC6 protein with Clb-CDC28 kinases has been reported in *S. cerevisiae* (15), we tested whether mitotic human cells contained a factor that inhibited the association of cyclin-cdk2 with GST-Cdc18 protein. The association of cyclin-cdk2 with Cdc18 protein was indeed specifically inhibited by the addition of a mitotic extract to an S-phase extract (Fig. 5B, middle blot; compare lanes 6 and 7 to lane 2). The inhibitor was specific for Cdc18-cdk2 association, because p21-cdk2 association was not inhibited (Fig. 5B, bottom blot). When the beads with GST-Cdc18 protein were preincubated with mitotic extracts, washed, and incubated with S-phase extracts, association with cdk2 was unaffected (data not shown), suggesting that the mitotic inhibitor

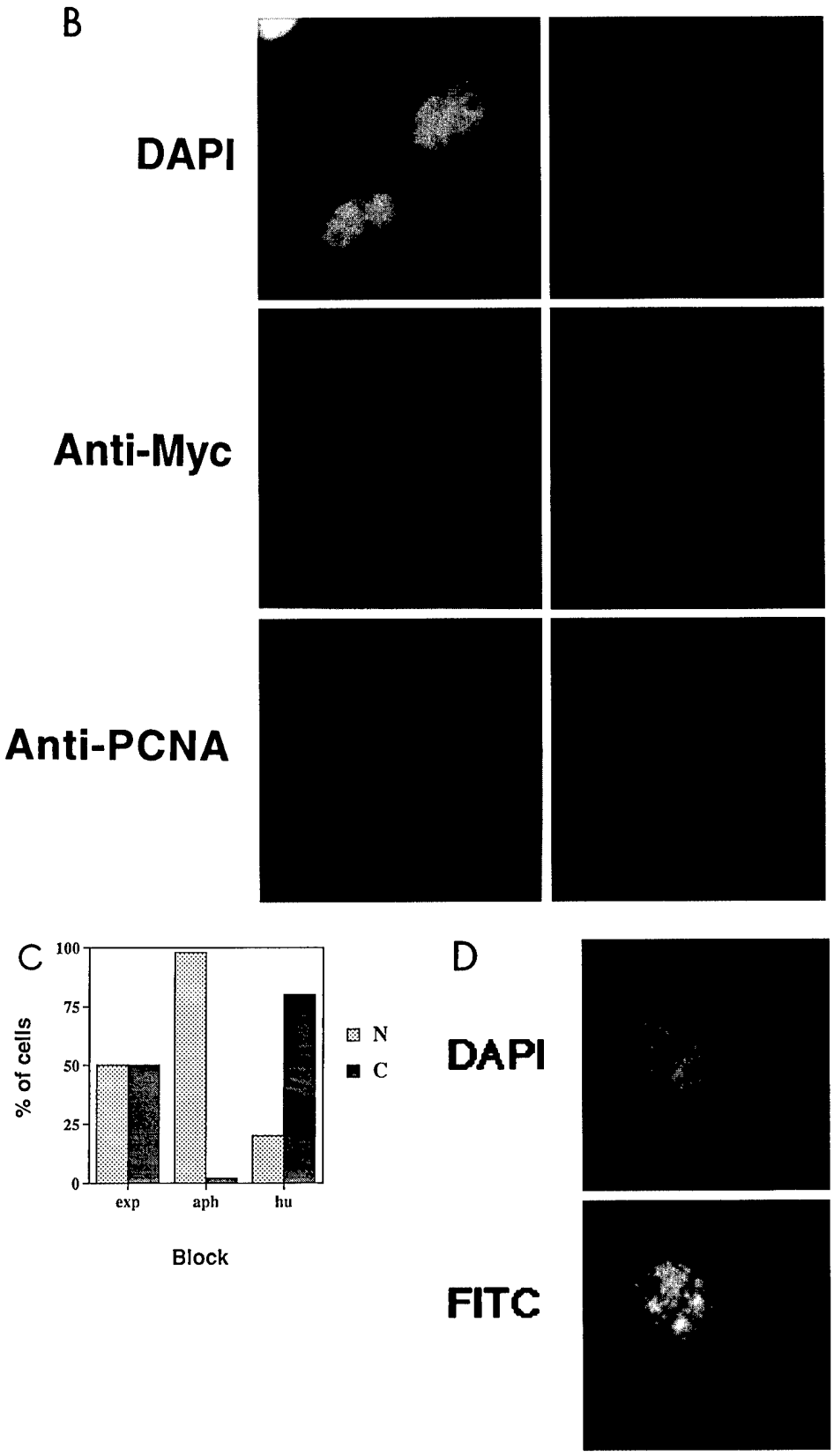

FIG. 3—*Continued.*

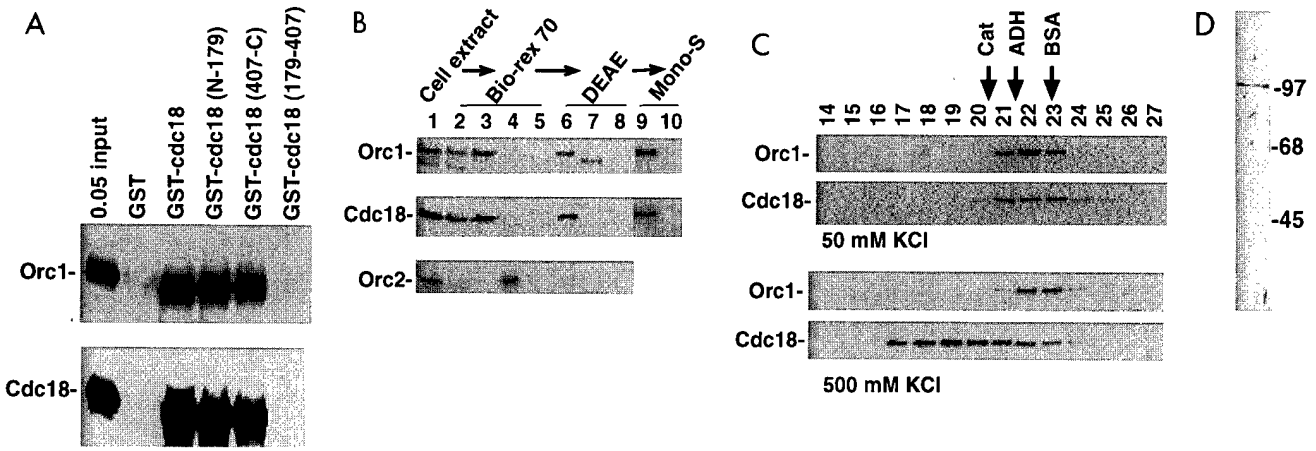

FIG. 4. Association of Cdc18 protein with Orc1 protein. (A) Glutathione-agarose beads coated with GST, GST-Cdc18 protein, or indicated deletion derivatives prepared in *Escherichia coli* were incubated with Orc1 protein or Cdc18 protein produced by in vitro transcription-translation in rabbit reticulocyte lysates. Bound proteins were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. (B) Immunoblots with anti-Orc1 protein, anti-Cdc18 protein, and anti-Orc2 protein antibodies. Lane 1, HeLa cell extract was fractionated over a Bio-Rex 70 column; lane 2, flowthrough of 0.15 M K acetate; lane 3, 0.3 M K acetate eluate containing 7% of input protein; lane 4, 0.6 M K acetate eluate; lane 5, 1.5 M K acetate eluate. The 0.3 M eluate was fractionated on DEAE-Sepharose. Lane 6, flowthrough at 0.2 M K acetate containing 57% of the input protein; lane 7, 0.35 M K acetate eluate; lane 8, 1 M K acetate eluate wash. The flowthrough in lane 6 was passed over a Mono-S column. Lane 9, flowthrough at 0.2 M KCl containing 25% of the input protein; lane 10, eluate at 0.6 M KCl. (C) Orc1 and Cdc18 proteins coelute as a heterodimer of 150 kDa upon gel filtration at 50 mM KCl (top) but are separated at 500 mM KCl (bottom). The flowthrough from the Mono-S column was loaded on a Superose 12 column. Fractions were immunoblotted with anti-Orc1 protein and anti-Cdc18 protein antibodies. The positions of elution of the molecular mass markers catalase (Cat; 300 kDa), alcohol dehydrogenase (ADH; 150 kDa), and bovine serum albumin (BSA; 68 kDa) are indicated. (D) Silver stain of the fraction containing Orc1 protein purified to homogeneity. The numbers indicate molecular masses in kilodaltons.

did not stably associate with or modify Cdc18 protein. Thus, the association of the S-phase activator cdk2 with Cdc18 is not only regulated by a requirement for cyclins but also inhibited by a factor present in mitotic cells, similar to the situation in *S. cerevisiae.*

In vivo association of hCdc18 with Orc1 and cyclin-cdk2. To demonstrate the association of Cdc18 protein with cyclin-cdk2 and with Orc1 protein in vivo, a plasmid expressing GST-Cdc18 protein was transiently transfected into human kidney 293T cells (Fig. 6). Negative controls included cells transfected with plasmids expressing GST or GST fused to a short protein expressed from the antisense strand of the Cdc18 gene (GST-Cdc18rev). Selective precipitation of GST-Cdc18 protein from the cell extracts showed coprecipitation of cyclin A, cdk2, and Orc1 protein, confirming that the cyclin-cdk2-Cdc18 protein and Orc1-Cdc18 protein associations are observed in cells. The addition of ethidium bromide (which intercalates into DNA) (21) did not affect the interactions (Fig. 6, lane 3), implying that the interactions are not mediated through DNA. Orc2 protein was not present in these precipitates (data not shown), confirming that Orc1-Cdc18 protein heterodimers are more stable in extracts of asynchronous cells than an ORC containing both Orc1 and Orc2 proteins.

DISCUSSION

We have identified a human CDC6/Cdc18 protein and shown that it associates directly with Orc1 protein and cyclin-cdk's. The protein is homologous to *S. pombe* Cdc18, *S. cerevisiae* CDC6, and human Orc1 proteins. The similarity in size of the protein to yeast CDC6/Cdc18 protein and the direct physical association with Orc1 protein and with cyclin-cdk's in vitro and in vivo support the hypothesis that this protein is the human equivalent of CDC6/Cdc18 protein. In the *S. cerevisiae* genome there are only two genes that belong to the Orc1-Cdc18 gene family. However, human cells may contain a larger family of Orc1-Cdc18 genes, and a better candidate for hCdc18 may emerge in the future. This caveat aside, we call the protein described in this paper hCdc18 protein.

Although in the yeasts the CDC6/Cdc18 protein is destroyed as cells enter S phase, the situation appears slightly different in humans. Williams and coworkers noted an absence of nuclear hCdc18 protein in serum-starved human WI38 fibroblasts and an increase followed by a decrease of nuclear hCdc18 protein as the cells passed through S phase. This result was postulated to be consistent with the rapid degradation of hCdc18 protein in G_2-M (36). However, they also noted the absence of any changes in nuclear Cdc18 protein levels as transformed HeLa cells passed through the cell cycle. In our experiments, which included several controls and were done in both transformed and untransformed cells (including WI38 fibroblasts), the level of total hCdc18 protein does not change significantly during the cell cycle. The protein is also abundantly present in G_0 (serum-starved) fibroblasts. Therefore, we postulate that the regulation of hCdc18 protein during the cell cycle is not achieved via changes in total protein level. The constant amount of Cdc18 protein despite the periodic expression of the RNA could be due either to the protein being long-lived or to an increase in the degradation of the protein to compensate for increased synthesis in S phase. An hCdc18 protein isoform of slightly reduced mobility was present in the cell extracts, and although considerably less abundant than the major protein band, increased in G_1 relative to G_0. Therefore, another possibility is that Williams and coworkers detected only this rare slower-moving isoform with their antibody (which was raised against an N-terminal portion of the protein) while we detected this and the more abundant hCdc18 protein with our antibody.

Changes in the subcellular localization of hCdc18 protein throughout the cell cycle provide a better explanation of the apparent discrepancy between the two reports. Recombinant epitope-tagged hCdc18 protein was seen in G_1 nuclei along with a dramatic change in its localization to the cytoplasm in S phase. This could be either because hCdc18 protein is exported

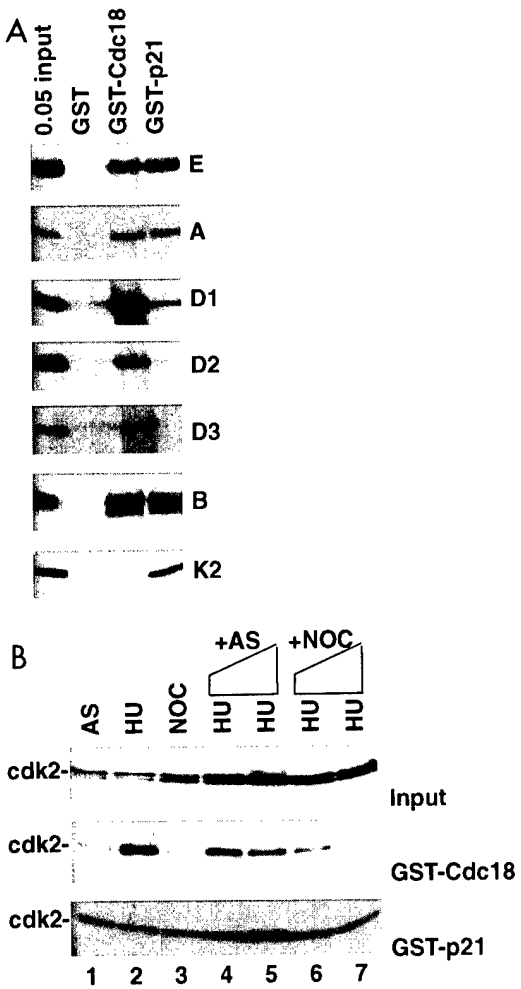

FIG. 5. Cdc18 protein associates with cyclin-cdk's through the cyclin, and the association is regulated by a mitotic inhibitor. (A) Indicated S^{35}-labeled proteins were produced by in vitro transcription-translation and tested for association with bacterially produced GST, GST-Cdc18, and GST-p21. 0.05 input, 5% of the input protein incubated with indicated glutathione-agarose beads. Proteins tested were cyclins E, A, D1, D2, D3, and B and cdk2 (K2). (B) Association of cdk2 with GST-Cdc18 is inhibited by a mitotic factor. The immunoblots show input cdk2 (top), cdk2 bound by GST-Cdc18 (middle), and cdk2 bound by GST-p21 (bottom). The extracts are from asynchronous (AS), hydroxyurea-blocked (HU), and nocodazole-blocked (NOC) HeLa cells. Lanes 1 to 3, 0.5-mg extracts directly incubated with glutathione-agarose beads coated with GST fusion proteins produced in bacteria; lanes 4 to 7, the same amount of HU extract used in lane 2 was incubated with increasing amounts of asynchronous cell extracts (lane 4, 0.5 mg; lane 5, 1 mg) or nocodazole-blocked mitotic cell extracts (lane 6, 0.5 mg; lane 7, 1 mg) before incubation with glutathione-agarose beads.

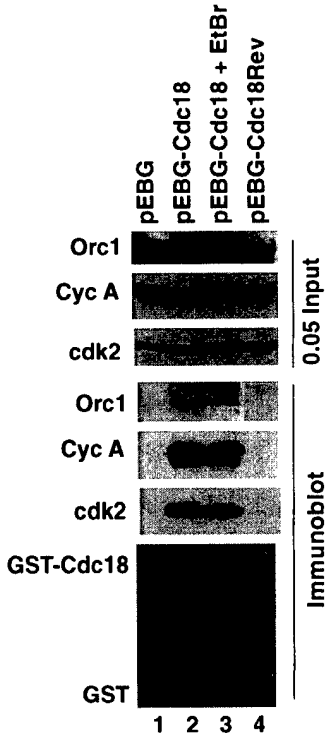

FIG. 6. Association of Cdc18 protein with Orc1 protein, cyclin A, and cdk2 in vivo. 293T cells were transfected with pEBG (lane 1), pEBG-Cdc18 (lanes 2 and 3), or pEBG-Cdc18rev (lane 4). Cellular proteins associated with glutathione-agarose beads were detected by immunoblot analyses. In the top three panels, lysates input into the reactions were detected by anti-Orc1 protein (Orc1), anti-cyclin A (Cyc A), and anti-cdk2 (cdk2) antibodies. In the bottom four panels, proteins associated with glutathione-agarose beads were detected by immunoblot analyses. To see the effect of ethidium bromide (EtBr) on the interaction, 50 μg/ml was added to the extract, incubated for 30 min on ice, and centrifuged to remove any particulate matter; the resulting extract was incubated with glutathione-agarose beads (lane 3). 0.05 Input, 5% of the input protein incubated with the indicated glutathione-agarose beads.

out of the nucleus into the cytoplasm in S phase or because the protein is selectively degraded in the nucleus while newly synthesized protein is denied entry into the nucleus and so accumulates in the cytoplasm. There is a parallel between our observations and that with *Xenopus* Cdc18 protein, which was displaced from the chromatin to perinuclear membrane vesicles during DNA replication (13). Human cells may regulate the functional pool of hCdc18 protein by selectively limiting its concentration in the nucleus once replication is initiated. The next round of replication then becomes dependent on the reentry of hCdc18 protein into the nucleus sometime before the next S phase.

We have also shown that the putative nuclear localization sequence in the N-terminal one-third of hCdc18 protein is not necessary for its nuclear localization. There are no other candidates for an NLS in the sequence of Cdc18 protein, making it likely that the protein is imported into the nucleus by its association with an unknown cellular factor which has its own NLS. Endogenous Cdc18 or Orc1 protein could be a candidate for the unknown cellular factor that chaperones the ectopic Cdc18 protein into the nucleus. Regulation of this association through the stages of the cell cycle may regulate the subcellular localization of Cdc18 protein.

Consistent with the role of Cdc18 protein as an adapter that recruits other DNA replication proteins to the chromatin-bound ORC (4, 13), we observed that the protein associates in vivo and in vitro with one of the components of the ORC, hOrc1 protein. In budding yeast, six polypeptides form a stable ORC which is resistant to high salt levels (2). Similar complexes were identified in other eukaryotes (14, 17, 22, 28). Transfection of epitope-tagged derivatives of hOrc1 and hOrc2 suggested that at least some hOrc1 and hOrc2 proteins are associated with each other (16). However, a human equivalent of the ORC has not yet been identified and our study suggests that endogenous hOrc1 protein is more stably associated with hCdc18 protein than with hOrc2 protein in human cell extracts. We cannot rule out the possibility that the lysis conditions disrupted a preexisting hOrc1-hOrc2 protein complex, that a small fraction of hOrc1 and hOrc2 proteins are associated with

each other, or that the hOrc1-hOrc2 protein association is strictly cell cycle regulated. However, under similar lysis conditions a detectable fraction of hOrc2 protein forms a stable complex with the third identified subunit of the human ORC, hOrc4 protein (32). Overall, the association of hCdc18 protein with hOrc1 protein appears more stable than that of hOrc2 protein with hOrc1 protein. It is possible that the interaction of the Cdc18-Orc1 protein heterodimer with hOrc2 protein (and the rest of the ORC) can be a regulated step during the human cell cycle.

The association of hCdc18 protein with cyclin-cdk2 is also consistent with the role of the former as an adaptor protein. Cyclin-cdk2, an activator of S phase, has been localized to replication foci (5), but how it is recruited there is unclear. The association of the kinase with hCdc18 protein provides an attractive mechanism by which cyclin-cdk's can be recruited to prereplication complexes in G_1. The mitotic inhibitor of this association could be important for preventing the inappropriate recruitment of the kinase in M phase. Elsasser and coworkers reported a similar mitotic inhibitor of the association of CDC6 with Clb-CDC28 kinases in *S. cerevisiae* and provided evidence that this inhibitor is Sic1, a protein that associates with the Clb-CDC28 kinase (15). At this moment we do not know the identity of the mitotic inhibitor of hCdc18-cyclin-cdk association.

In summary, the interprotein associations reported here strongly suggest that the protein we have identified is hCdc18 protein. The constant level of the protein throughout the cell cycle is difficult to reconcile with the existing model of how global degradation of human CDC6/Cdc18 protein regulates its activity. Instead, the results point to two other mechanisms by which hCdc18 protein activity could be regulated to limit the formation of prereplication initiation complexes to once per cell cycle in G_1. First, Cdc18 protein is exported from the nucleus (or selectively degraded in the nucleus while new protein accumulates in the cytoplasm) after origins have fired in S phase, necessitating a reentry step later in the cell cycle before the nucleus can replicate again. Second, if the interaction between Cdc18 protein and the S phase-promoting factor cyclin-cdk is essential for the initiation of DNA replication, the mitotic inhibitor of this interaction could prevent a premature interaction until the appropriate time in G_1. Future work will be directed towards testing these hypotheses.

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